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Properties of a Novel Early Lymphoid Activation Gene (Elag) Isolated from a Hodgkin's-Disease Cell Line

John Scott Bennett
Loyola University Chicago

LOYOLA UNIVERSITY OF CHICAGO

PROPERTIES OF A NOVEL EARLY LYMPHOID ACTIVATION GENE (ELAG) ISOLATED FROM A HODGKIN'S-DISEASE CELL LINE

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JOHN SCOTT BENNETT

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DEDICATION

For my wife, Connie.

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LIST OF ABBREVIATIONS

anti-CD3	anti-human Leu 4 monoclonal antibody
CD28 RE	CD28 receptor element
cDNA	Complementary DNA
COIII	Cytochrome oxidase III
DEPC	Diethyl pyrocarbonate
DTT	Dithiotreitol
EBV	Epstein-Barr virus
EBNA	Epstein-Barr nuclear antigen
ELAG	Early lymphoid activation gene
H/RS	Hodgkin's/Reed-Sternberg
IL	Interleukin
INR	Initiator region
LMP	Latent membrane protein
NLS	Nuclear localization signal
PBMC	Peripheral blood mononuclear cell
PCR	Polymerase chain reaction
PHA	Phytohemagglutinin
PMA	Phorbol 12-myristate 13-acetate (also TPA)
RACE	Rapid Amplification of cDNA Ends
RT	Reverse transcription
RT/PCR	Reverse transcription/polymerase chain reaction
TdT	Terminal deoxynucleotidyl transferase
TPA	12-O-tetradecanoyl phorbol-13-acetate (also PMA)
TRE	TPA response element

INTRODUCTION

Our laboratory has been interested in characterizing the molecular etiology of Hodgkin's disease. Hodgkin's disease is an enigma since its description decades ago in that little is known of the origin of the malignant cell, the etiology and pathology of the disease, nor about the molecular basis of tumorigenic transformation. Because of this lack of knowledge, diagnosis of the disease is difficult, often leading to inappropriate treatment. A better understanding of this disease, particularly at the molecular level, would help in the management of Hodgkin's disease.

Recently, several groups showed that the Epstein-Barr virus (EBV) genome is in the nucleus of Hodgkin's/Reed-Sternberg cells, suggesting a direct correlation between EBV infection and the tumorigenic transformation of cells to the Hodgkin's disease phenotype. Based on these observations, I hypothesized that the oncogenic events responsible for EBV associated and non-EBV associated Hodgkin's disease are mediated by aberrant expression of cellular genes which are also aberrantly expressed in EBV infected non-Hodgkin's lymphoma. A key EBV encoded gene involved in immortalization and potentially in

transformation is the Epstein-Barr nuclear antigen 2 (EBNA 2). My objective therefore was to isolate novel cellular genes over-expressed in Hodgkin's-disease cells and which are potential targets of EBNA 2 in EBV infected cells. To do so, I developed a differential screening procedure to isolate such genes in an EBV negative Hodgkin's disease cDNA library that hybridize to probes from EBNA 2 expressing African Burkitt's lymphoma cell line, but which do not hybridize with probes from an African Burkitt's lymphoma cell line lacking EBNA 2.

The result of the screening procedure was the isolation of a novel cDNA that we named ELAG (Early Lymphoid Activation Gene) based on its transient expression during activation of peripheral blood mononuclear cells (PBMC) with either phytohemagglutinin (PHA) or anti-CD3 monoclonal antibody. Analysis of the open reading frame suggests that ELAG may encode a nuclear phosphoprotein. I hypothesize that ELAG expression may be required for the activation of other genes involved in the T cell activation cascade. Because ELAG is tightly regulated during the activation and proliferation of normal lymphoid cells, it would therefore be an ideal candidate gene as a novel oncogene that when over-expressed may provide aberrant signals that are involved in tumorigenic transformation.

Further investigation of ELAG may provide a better understanding of the poorly understood yet highly intricate regulation of T cell activation during the immune response. Additionally, characterization of the normal function of ELAG may provide clues about its potential role in tumorigenesis.

REVIEW OF RELATED LITERATURE

I. Overview of the disease

Hodgkin's disease was originally characterized in 1832 by Thomas Hodgkin's in a paper describing the clinical histories and postmortem observation of six people with enlarged lymphatic glands (Kaplan, 1980). Seventy years later Carl Sternberg and Dorothy Reed described the characteristic giant multinucleate cells in the diseased region of a Hodgkin's disease tumor. This malignant cell of Hodgkin's disease is now known as the Reed-Sternberg cell. In addition to the Reed-Sternberg cell, a mononuclear variant cell is often identified in diseased tissue. The distinction between the mono- and multi-nuclear cells is unclear and potentially represent the same cell type. Therefore, for the clarity in this thesis the malignant cell in Hodgkin's disease tissue will be referred to as the Hodgkin's/Reed-Sternberg cell (H/RS) which refers to both the mono- and multinuclear cell.

The definition of Hodgkin's disease as a malignancy has been controversial in the past because of the low abundance of H/RS cells in the tumor (Kaplan, 1980). The H/RS cell often comprises less than 1% of the tumor mass

while most of the tumor is comprised of non-neoplastic infiltrate (Anastasi *et al.*, 1987; Cossman *et al.*, 1988). The malignant nature of Hodgkin's disease was questionable because of its pathology. Close analysis of the H/RS cells reveals characteristics similar to other tumor cells. H/RS cells in diseased lymph nodes are often characterized by aneuploidy and clonality (Thangavelu and Le Beau, 1989). Additionally, tumors produced by heterotransplantation into nude mice strongly resemble Hodgkin's disease, a finding which supports the contention that Hodgkin's disease is a malignancy (Diehl *et al.*, 1982; Schaadt *et al.*, 1979).

Hodgkin's disease is characterized by a defect in cellular immunity whereas humoral immunity remains unimpaired (Twomey and Rice, 1980; Bjorkolm *et al.*, 1982; Fisher, 1982). Patients with Hodgkin's disease have an increased incidence of infection with opportunistic pathogens including fungi, viruses, and tuberculosis (Slivnick *et al.*, 1989). The H/RS cell is in an activated state and secretes lymphokines which affect the immune system (Borowitz *et al.*, 1982; Maggi *et al.*, 1988; Poppema *et al.*, 1982). Patients in advanced stages of Hodgkin's disease die of severe immunosuppression.

The etiology of Hodgkin's disease is poorly understood. Investigations by MacMahon showed a bimodal age-specific incidence curve (MacMahon, 1957). Analysis

of the incidence curve shows a specific incidence peak for individuals between the ages of 15 to 45. Other investigators suggested that Hodgkin's disease in younger patients tends to be an inflammatory disease whereas Hodgkin's disease in older patients is more similar to a neoplasm. MacMahon therefore suggested that Hodgkin's disease may not be a single entity but a syndrome comprising at least two entities with different etiologies.

My goal was to investigate the molecular etiology of Hodgkin's disease. The impetus for the initiation of this project stemmed from the lack of knowledge regarding the cellular and molecular basis of Hodgkin's disease. Because of this lack of information, diagnosis and treatment are often difficult. On the other hand, Hodgkin's disease is curable when diagnosed early enough and chemotherapy is initiated. Therefore, a better understanding of the molecular etiology might help us understand how various drug therapies work and how to improve them. The discussion in this Literature Review section addresses the current status of information regarding Hodgkin's disease. Additionally, I have reviewed the recent literature on Epstein-Barr virus (EBV) and its association with the Hodgkin's disease phenotype that led to the initiation of this project.

II. Characterizing and defining the Hodgkin's-disease cell

Hodgkin's disease remains an enigma in that it is very poorly characterized. Analysis of the histological type and the cellular origin of the Hodgkin's-disease cell has demonstrated that Hodgkin's disease is more characteristic of a diverse range of cancers than of a single disease entity.

A. Histopathology reveals a diversity of transformed cells in Hodgkin's disease tissue

Hodgkin's disease is a diverse disease, and therefore, pathologists have attempted to morphologically and prognostically categorize the heterogeneous nature of Hodgkin's disease. Four histopathological classifications have been proposed to categorize Hodgkin's disease tumors; nodular sclerosis, mixed cellularity, lymphocytic predominance, and lymphocytic depleted (Lukes, 1971; Lukes and Butler, 1966a; Lukes *et al.*, 1966b). The histologic subtype is not an independent prognostic factor if the disease is treated appropriately.

Unequivocal diagnosis of Hodgkin's disease can only be made by microscopic examination of one or more tissue specimens (Anastasi *et al.*, 1989). However, it is often difficult to make an accurate characterization and diagnosis of the disease due to many factors specific to

Hodgkin's disease. First, as described earlier, the malignant cell often comprises a very low percentage of the actual tumor mass (Anastasi *et al.*, 1987; Cossman *et al.*, 1988). Second, there are different types of H/RS cells. Malignant cells in a Hodgkin's disease tumor are either mononuclear cells or contain multiple nuclei. Additionally, cells similar to the H/RS cells are often found associated with other pathological events such as inflammatory lymphadenopathy and infectious mononucleosis. Therefore, it is difficult to diagnose Hodgkin's disease specifically based on the observation of a cell that looks like a H/RS cell in a biopsy (Anastasi *et al.*, 1989).

B. Origin of the Hodgkin's/Reed-Sternberg cell

The precursor cell of Hodgkin's disease is unknown. Investigators have attempted to characterize the cellular origin of the H/RS cell in lymphoid tissue and in cell culture lines by utilizing immunohistochemical staining and morphologic analysis. Conflicting reports describe the H/RS cell as a B lymphocyte (Taylor, 1974; Boecker *et al.*, 1975; Linch *et al.*, 1985; Pinkus and Said, 1988), T lymphocyte (Andreesen *et al.*, 1984; Kadin, 1985), monocyte-macrophage (Strauchen, 1984; Kadin, 1993; Andreesen *et al.*, 1989; Foon and Todd, 1986; Payne *et al.*, 1982), or as originating from an interdigitating reticulum cell (Hansmann and Kaiserling, 1982; Kadin, 1982).

Morphologic analysis of Hodgkin's disease lymph nodes revealed ultrastructural similarities between H/RS cells and either lymphocytes or interdigitating reticulum cells. Both interdigitating reticulum cells and H/RS cells function as antigen presenting cells, and lack differentiation antigens characteristic of monocytes, and pre-B or pre-T cells (Kadin, 1982; Stein *et al.*, 1982). However, interdigitating reticulum cells fail to stain with monoclonal antibodies specific for surface markers of H/RS cells, including HeFi-1 or Ki-1 (Schwarrting *et al.*, 1987; Stein *et al.*, 1982). Additionally, H/RS cells fail to stain with monoclonal antibodies which specifically bind reticulum cells including OKT6 (Stein *et al.*, 1982; Stein *et al.*, 1983).

Investigations demonstrating that H/RS cells positively stain for alpha-1 antitrypsin, alpha-1 antichymotrypsin, acid phosphatase, and Fc receptors led some investigators to suggest that H/RS cells originate from a monocyte/macrophage cell lineage (Strauchen, 1984; Kadin, 1993; Andreesen *et al.*, 1989; Foon and Todd, 1986; Payne *et al.*, 1982). However, alpha-1 antitrypsin is also expressed in activated lymphoblasts (Payne *et al.*, 1982; Jones *et al.*, 1986). Further, H/RS cells do not react with monocyte specific antibodies and lack phagocytic properties characteristic of macrophages (Stein *et al.*, 1982; Dorreen *et al.*, 1984; Stein *et al.*, 1986).

Conversely, monocytes and macrophages do not react with the Ki-1 monoclonal antibody (Jones *et al.*, 1986; Schwarrting *et al.*, 1987; Stein *et al.*, 1983).

Many recent investigations support the notion that H/RS cells originate from lymphocytes. Activated T cells react with the monoclonal antibody anti-Leu-M 1, which typically stains H/RS cells (Schwarrting *et al.*, 1987; Timens et al., 1986). The presence of IL-2 (interleukin 2) receptors further support the contention that H/RS cells are of lymphoid origin (Schwarrting *et al.*, 1987). Additionally, lymph node specimens have revealed the presence of H/RS cells that react with pan B monoclonal antibodies (Pinkus and Said, 1985; Timens *et al.*, 1986).

An analysis of H/RS cells in tissue culture indicates that there may be spectrum of cell types involved in the development of Hodgkin's disease. One study showed that one Hodgkin's-disease cell line, L428 is comprised of three morphologically distinct cells; larger multinucleate Reed-Sternberg cells and medium size mono- or bi-nucleated cells (Schaadt *et al.*, 1979; Diehl *et al.*, 1981). It is likely that the different subtypes of Hodgkin's disease arise from different progenitor cells types (Slivnick *et al.*, 1989). Efforts to characterize the lineage of the H/RS cell will continue with the hope of improving the efficiency of diagnosis.

Several cellular genes are over-expressed in association with Hodgkin's disease. These genes include CD30, CD15, CD74, CDw70 and interleukin-6 (IL-6) and IL-9. However, these genes are expressed in a wide range of tumor cell types and therefore there specific role in the development of Hodgkin's disease is unclear. Additionally, the cell surface markers are not consistently expressed on all H/RS cells. One study showed that CD30 is expressed in H/RS cells of approximately 70% of the Hodgkin's disease nodes tested and CD15 was detected on only 65% of the diseased nodes tested (Chu *et al.*, 1992). To date, there is no consensus immunophenotype of the H/RS cell.

C. Molecular basis of Hodgkin's disease

1. Cytogenetic abnormalities

Efforts to characterize the origin of the H/RS cell have led to the identification of cytogenetic abnormalities that are potentially specific for Hodgkin's disease. To better understand the molecular biology of Hodgkin's disease and to provide data about the oncogenic events, several investigators have tried to characterize molecular anomalies that correlate with the transformed phenotype. Unique cytogenetic abnormalities that demonstrate a high correlation with Hodgkin's disease or Hodgkin's disease subtype have not been identified

(Thangavelu and Le Beau, 1989). This is in sharp contrast to other hematopoietic tumors such as Burkitt's lymphoma and chronic myelogenous leukemia which demonstrate distinct chromosomal alterations which are considered as molecular hallmarks of these disease types. Burkitt's lymphoma is characterized by a translocation of the c-*myc* proto-oncogene with human immunoglobulin heavy chain [t(8;14], kappa light chain [t(8;2], or the lambda light chain genes [t(8;22] (Della-Favera *et al.*, 1982; Taub *et al.*, 1982; Della-Favera *et al.*, 1983; Adams *et al.*, 1983; Hollis *et al.*, 1984; Davis *et al.*, 1984; Pelicci *et al.*, 1986). The translocation results in the constitutive expression of the c-*myc* gene (Croce and Nowell, 1985; Croce, 1987; Bishop, 1987). CML is characterized by the observation of the Philadelphia chromosome in which the c-abl gene found on chromosome 9 is fused with the coding sequences of the *bcr* gene (Rowley, 1973; de Klein *et al.*, 1982). The resulting fusion gene is translated as a chimeric protein with enhanced activity (Konopka and Witte, 1985; Westbrook, 1988). However, the number of cases studied may be too few to make any conclusions. Further study of the H/RS cell may provide answers that will eventually allow for the use of cytogenetic aberrations as a prognostic marker, such as those identified with non-Hodgkin's lymphomas.

Anueploidy, or altered diploid number, is a common characteristic of the H/RS cell (Fleishmann *et al.*, 1976; Hansmann *et al.*, 1986; Hess *et al.*, 1986; Kristoffersson *et al.*, 1987; Reeves and Pickup, 1980; Seif and Spriggs, 1967; Slavutsky *et al.*, 1985). Increased copy number of chromosome 5 was characterized in over 25% of cases studied (Thangavelu and Le Beau, 1989). Additionally, a gain of chromosomes 1,2,9,11,12,16,18,20 and 21 occurred in at least 10% of the cases. The loss of chromosomes 10, 13, 17, 21 and 22 were observed in nearly 10% of all cases. Loss of chromosome 3 was never observed.

A review of the same cases described above showed that structural alterations were identified which involved chromosome arms 1p (25%), 1q (25%), as well as chromosome arms 2q (16%), 6q (21%), 11p (12%), 11q(12%), and 14q (12%). Aberrant chromosomal banding patterns were frequently found at positions 1p21, 5p15, 11p13, 14q32 and 18p11. As discussed above, aberrations of chromosome band 14q32 are frequently found in B cell neoplasms (over 70% of the cases), suggesting a B cell origin for these particular cases of Hodgkin's disease.

In the study described here, I characterize a novel gene from the Hodgkin's-disease cell line L428, which might be deregulated in this cell type. An understanding of the karyotype of the L428 cell may eventually be important in the interpretation of the results of the

study described here because genes over-expressed in these cells could potentially be deregulated due to a translocation event. The specific chromosomal aberrations identified in the L428 were found at positions 2q33, 7q22-36, 11q21/23, 14q32, and 21q21-22. As discussed above, aberrations at position 7q35 are found at high frequencies in T cell tumors while aberrations at position 14q32 are frequently identified with B cell lymphomas.

2. Oncogenes in Hodgkin's disease

It is suspected that activation of proto-oncogenes may be involved in the development of Hodgkin's disease. Perhaps a translocation, as discussed above, may be responsible for activating a proto-oncogene. Other potential mechanisms that may be involved include point mutations (Bishop, 1987), over expression due to gene amplification (Bishop, 1987; Marcu *et al.*, 1992), or inactivation of a tumor suppressor gene such as the retinoblastoma gene (Rb) (Levine, 1993).

The difficulty in characterizing gene expression in the H/RS cell of a diseased lymph node stems from the low proportion of tumor cells to normal tissue. Therefore, several groups analyzed H/RS cell lines to determine if any proto-oncogenes are consistently activated.

Jucker *et al.* analyze the expression of 20 proto-oncogenes in four H/RS cell lines (L428, L540, CO, and DEV) compared to their expression in normal hematopoietic

cell (Jucker *et al.*, 1990). They found that c-*myc*, p53, c-*jun*, *pim*-1, p56-*lck*, c-*syn*, c-*raf* and N-*ras* were each expressed in at least three of the four cell lines tested. Additionally, they observed that N-*myc* and p56-*lck* were only expressed in the CO cell line which also expresses T cell receptor genes.

Specifically, the analysis of oncogene expression in the L428 Hodgkin's-disease cell line showed that c-*myc*, c-*jun*, c-*syn*, c-*myb*, c-*met*, *pim*-1, c-*raf* and p53 demonstrate normal levels of expression (Jucker *et al.*, 1990). Other proto-oncogenes tested including N-*myc*, L-*myc*, c-*fos*, c-*src*, c-*fgr*, p56-*lck*, c-*mos* were not detected. Additionally, no mutations were identified in any of these genes. However, aberrant transcripts from the c-*fes* gene were identified. No point mutations were found in the Ki-*ras*, H-*ras* or the N-*ras* gene.

More recent studies have used <u>in situ</u> hybridization and immunohistochemical techniques to directly analyze gene expression in the H/RS cells in diseased tissue. One study has shown that p53 is over-expressed in the H/RS cell in 32% of the cases analyzed (Niedobitek *et al.*, 1993). Their investigation did not reveal any specific mutations in the gene but instead suggest that over-expression of this tumor suppressor gene may be an indicator of altered wild-type p53 function. Two additional studies suggest a role of activated *bcl*-2 (Jiwa

et al., 1993; Reid *et al.*, 1993). One study showed that the both c-*myc* and *bcl*-2 are expressed in the H/RS cell in 72% of samples tested and suggested that these two oncogenes may act in concert to affect the pathogenesis of Hodgkin's disease (Jiwa *et al.*, 1993).

III. Epstein-Barr virus (EBV) and Hodgkin's disease

Since the original discovery of the virus by Epstein, Barr and Achong (Epstein *et al.*, 1964; Epstein and Barr, 1964; 1986), the Epstein-Barr virus (EBV) was found to cause infectious mononucleosis and has become associated with the development of Burkitt's lymphoma (Epstein *et al.*, 1964; Epstein and Barr, 1964; 1986), nasopharyngeal carcinoma (Hirayama and Ito, 1981; Fahraeus *et al.*, 1988; Chan *et al.*, 1989; Tomei *et al.*, 1987; Young *et al.*, 1988) and lymphoproliferative diseases induced in immunosuppressed patients (Cen *et al.*, 1993; Seiden and Sklar, 1993; Purtilo *et al.*, 1992). It has long been suggested that the Epstein-Barr virus (EBV) may also be involved in the development of Hodgkin's disease. Epidemiological studies supported this contention by identifying a higher incidence of Hodgkin's disease among people who had been afflicted with infectious mononucleosis (Kaplan, 1980). However, the association of

EBV and Hodgkin's disease, without direct evidence, remained questionable.

Studies by Sklar and colleagues showed in southern blot analysis of biopsy tissue from Hodgkin's disease patients that the EBV genome is harbored in the tumor mass (Weiss *et al.*, 1987). However, because the H/RS cells generally makes up less than 1% of the tumor mass, these studies did not conclusively demonstrate the presence of EBV in the H/RS cells. These findings could have been the result of an EBV infected B cell population in the lymph node.

A later study by this same group utilized <u>in situ</u> hybridization to directly demonstrate that the EBV genome was harbored in the H/RS cell of disease lymph nodes in 20% of the Hodgkin's-disease patients tested (Anagnostopoulos *et al.*, 1989; Weiss *et al.*, 1989). These findings supported the contention that EBV may be involved in the development of Hodgkin's disease. Similar studies by other investigators showed that the EBV genome is harbored in the H/RS cell of potentially 60% of the patients tested (Staal *et al.*, 1989; Libetta *et al.*, 1990). One study which utilized PCR to detect EBV claimed that as many as 78% of diseased nodes contained the EBV genome (Herbst *et al.*, 1990). However these results are dubious in that the sensitivity of PCR may allow for the

detection of EBV in a small population of contaminating non-H/RS cells.

I was interested in the association between EBV infection and the development of Hodgkin's disease and hypothesized that the oncogenic events responsible for Hodgkin's disease, both EBV associated and EBV negative independent Hodgkin's disease, are mediated by aberrant expression of cellular genes which are also aberrantly expressed in EBV infected non-Hodgkin's lymphoma. My objective was to develop a screening procedure to isolate cellular genes that may be affected by the presence of EBV and that contribute to the transformed phenotype of Hodgkin's disease. In order to describe the screening method that I utilized, it is necessary to review the molecular biology of the Epstein-Barr virus and what is known about the role of EBV in tumorigenic transformation.

IV. Molecular basis for EBV immortalization and transformation

A. The EBV genome

The Epstein-Barr virus was originally identified and isolated from African Burkitt's lymphoma tumors (Epstein *et al.*, 1964; Epstein and Barr, 1964; 1986). The EBV genome of the virus is a double stranded DNA molecule of

approximately 173 kb (Baer *et al.*, 1984). It has been cloned as a set of overlapping fragments of the restriction enzymes *Eco*R I and *Bam*H I and regions of the genome are generally referred to by the name of the corresponding *Bam*H I fragment. The EBV genome potentially encodes over 100 genes, however, the virus remains in a latent state while expressing only a few EBV encoded genes. These include the following: the Epstein-Barr nuclear antigens [EBNA] 1, 2a, 2b, 3a, 3b, 3c, 4, 5, and 6; the leader peptide, LP; the latent membrane proteins [LMP] 1, 2a and 2b; and the EBV encoded RNAs EBER 1 and EBER 2. One gene recently described that is expressed during the lytic phase is the EBV replication activator ZEBRA (Baumann *et al.*, 1993).

B. Expression of EBV encoded genes during latent infection

1. LCL phenotype

The in vitro EBV-infected B lymphocytes are immortalized into lymphoblastoid cell lines (LCL) which have gained the ability to proliferate in culture indefinitely (Baichwal and Sugden, 1988; Rogers *et al.*, 1992; Middleton *et al.*, 1991; Klein, 1989). These cells usually do not produce virus particles. Instead the virus remains in a latent state with only a small number of EBV encoded genes being expressed. Much of our understanding about the pattern of EBV gene expression and the function

of EBV encoded genes has come from studies of EBV infected LCLs.

EBNA 1 is required for replication of the virus (Yates *et al.*, 1985; Lupton and Levine, 1985). This is the only viral protein consistently expressed in EBV immortalized cells. This protein specifically binds to sequences at the origin of replication, to facilitate replication (Lupton and Levine, 1985). EBNA 1 has also been shown to transactivate viral transcription from the *Bam*H I K-promoter of the EBV genome (Sugden and Warren, 1989).

The role of EBNA 3a, 3b, 3c, 4, 5 and 6 are poorly understood (Rogers *et al.*, 1992). However EBNA 3 proteins have an affinity for single stranded DNA (ssDNA) while EBNA 4 has an affinity for both single stranded DNA (ssDNA) and double stranded DNA (dsDNA) (Rowe *et al.*, 1989; Rogers *et al.*, 1992). The function of the non-coding EBERs is unclear, but the presence of EBER is recognizes as the most consistent indicator of latent EBV infection (Raab-Traub and Flynn, 1986). Additionally the roles of the EBV encoded products in the development of the transformed phenotype has not been distinguished.

Other latent proteins include EBNA 2, LMP, and the *Bam*H I A reading frame encoded proteins. Because of the association between these proteins and the ability of EBV

to immortalize and transform cells, these proteins will be discussed in a section addressing this important aspect.

The ZEBRA protein is translated from transcripts from the EBV *Bam*H I Z-fragment (BZLF 1). Expression of this protein alone is sufficient to activate the lytic cycle of EBV infection (Baumann *et al.*, 1993). ZEBRA shares homology with AP-1 transcriptional factors including Fos, Fra-1, Jun, and Jun-B (Carey *et al.*, 1992; Sato *et al.*, 1992). This EBV encoded protein is able to bind to the AP-1 site of the c-*fos* promoter and induce expression of c-*fos* (Carey *et al.*, 1992). This protein binds the canonical TRE/AP-1 site and therefore is potentially able to transactivated several cellular genes.

2. Burkitt's lymphoma

EBV was first discovered in a cell line derived from Burkitt's lymphoma tissue (Epstein *et al.*, 1964; Epstein and Barr, 1964; 1986). The pattern of latency observed in these cells in greatly restricted in that only EBNA 1 is expressed (Rowe *et al.*, 1987; Gregory *et al.*, 1990) when the cell lines are first established in culture. Continued passage of these cells results in the progression from this restricted pattern of latency to a phenotype that more closely resembles an LCL. When first in culture, Burkitt's lymphoma cell lines are classified as type 1 (Burkitt's lymphoma type) with a restricted latency. Type 2 cells have an intermediate latency

pattern of EBV gene expression and type 3 develop an LCL-like phenotype (Altiok *et al.*, 1992; Rowe *et al.*, 1987; Gregory *et al.*, 1990). Type 3 Burkitt's lymphoma cell express most EBV latent proteins as well as cellular antigens characteristic of an LCL.

3. Nasopharyngeal carcinoma and Hodgkin's disease

A third pattern of latency is exhibited in nasopharyngeal carcinoma cells. These cells express EBNA 1, LMP and transcripts originating from the *Bam*H I open reading frame (Gilligan *et al.*, 1990). Recent studies have shown by in situ hybridization that EBV infected H/RS cells express the same EBV encoded genes (Deacon *et al.*, 1993). The role of these transcripts and the proteins that they potentially encode is not known.

C. The role of EBV encoded genes in immortalization and transformation

Although the ability of EBV to tumorigenically transform human cells is likely due to the coordinate activity of the latent proteins described above, the direct immortalization and transformation is likely triggered by two EBV encoded proteins; the Epstein-Barr virus nuclear antigen 2 (EBNA 2) and the latent membrane protein family (LMP 1, LMP 2a and LMP 2b). The recently identified transcripts originating from the *Bam*H I A-fragment may also be involved in transformation.

1. *Bam*H I A-open reading frame

A family of transcripts expressed in NPC cells, originating from the *Bam*H I A-region of the EBV genome have recently been characterized (Gilligan *et al.*, 1990). The proteins translated in vitro from these transcripts, 32-kDa, 26-kDa, 17-kDa, 15-kDa, and 11-kDa, are poorly characterized and their functions are unknown. Similar transcripts have been identified in the H/RS cells infected with EBV (Deacon *et al.*, 1993).

2. LMP proteins

The LMP is transcribed as a 2.6-kb RNA which is spliced as a 2.4-kb mRNA containing a 1.3-kb open reading frame. The translated protein has six hydrophobic transmembrane domains. The protein is translated as a 58-kDa protein. Analysis shows that the LMP proteins form patches on the cell surface indicating that it may be part of a complex of proteins that may be necessary for immortalization.

LMP 1 has been characterized as a true oncogene (Moorthy and Thorley-Lawson, 1992; Niedobitek *et al.*, 1992; Wang *et al.*, 1990). Rodent fibroblast transfected with this gene loose anchorage dependence acquire a transformed phenotype (Wang *et al.*, 1985; Wang *et al.*, 1988; Wang *et al.*, 1985). LMP 1 has been shown to act with EBNA 2 to cooperatively induce expression of cellular genes (Wang *et al.*, 1990). Despite the clear results of

tissue culture studies, the biochemical function of LMP in human cells and the development of human tumors is unclear. LMP 1 expression will induce the expression of bcl-2 resulting in the protecting the cell from apoptosis (Henderson *et al.*, 1991).

LMP 2A and 2B have recently been identified (Longnecker and Kieff, 1990). These two proteins are translated from alternatively spliced transcripts which are translated as nearly identical proteins except that the LMP 2A protein contains an additional 119 amino acids at its amino terminus (Peng and Lundgren, 1993). LMP 2A has been found to co-localize to the cellular membrane with LMP 1 (Longnecker and Kieff, 1990). The importance of this association is unclear. The association of LMP 2B with LMP 1 is only speculative at this time. The function of these two proteins are unknown.

3. EBNA 2

EBNA exists as two antigenically different proteins, EBNA 2A and EBNA 2B, which share 50% amino acid homology (Dambaugh *et al.*, 1984; Addinger *et al.*, 1985). The majority of EBV strains characterized to date encode EBNA 2A while EBNA 2B appears to be encoded by an EBV strain which is restricted to areas of equatorial Africa (Rowe *et al.*, 1989). Because EBNA 2A is the better characterized protein, and in much of the literature EBNA 2 is

synonymous with EBNA 2A unless otherwise mentioned, only EBNA 2A will be addressed.

EBNA 2 is a key determinant of lymphocyte transformation (Cohen *et al.*, 1989; Cohen *et al.*, 1992). This protein shares homology with the three viral oncogenes; adenovirus E1A, human papilloma virus-16 E7 and SV40 large T antigen (Harada and Yanagi, 1991). EBNA 2 is a transactivator of viral genes such as LMP 1 (Reisman and Sugden, 1986; Tsang *et al.*, 1991; Tsang *et al.*, 1991; Wang *et al.*, 1991b; Abbot *et al.*, 1990) and cellular genes (Cohen and Kieff, 1991; Rogers *et al.*, 1992). Infection of B cells with EBV results in the expression of CD23 (Wang *et al.*, 1990; Wang *et al.*, 1987; Wang *et al.*, 1991a), CD21 (Calender *et al.*, 1987), CD30 (Calender *et al.*, 1987), Bac-1 (Calender *et al.*, 1987), and c-*fgr* (Knutson, 1990). Cells transfected with EBNA 2 demonstrate obvious changes observed during EBV infection including increased growth in clumps.

The role of EBNA 2 in immortalization was clearly demonstrated in studies comparing the viral strains of two African Burkitt's lymphoma cell lines; Jiyoye and P3HR-1. The Jiyoye cell line was developed from the tumor cells of an African Burkitt's lymphoma. The cell is infected with a strain of EBV that encodes EBNA 2B (Hinuma and Grace, 1967). Virus isolated from tissue culture supernatants of Jiyoye cells can infect peripheral blood B lymphocytes and

result in the development of an immortalized cell line. The P3HR-1 cell line is a tissue culture derivative of the Jiyoye cell line (Hinuma and Grace, 1967). The P3HR-1 viral strain, which lacks the gene encoding EBNA 2 due to a spontaneous deletion, has lost the ability to immortalize cells in culture (Jeang and Hayward, 1983; Bornkamm *et al.*, 1982).

It is hypothesized that EBNA 2 immortalizes cells by inducing aberrant expression of cellular genes like CD30 and CD23 (Cordier *et al.*, 1990; Calender *et al.*, 1987; Wang *et al.*, 1987). Furthermore, it is likely that these cellular genes required for immortalization are expressed in Jiyoye cell line but that the P3HR-1 cells no longer expresses these cellular genes (Murray *et al.*, 1988).

Based on this information, I developed a differential screening method that would target cellular genes, like CD23 and CD30 or any normal gene that is a target of EBNA 2 and that is expressed in Hodgkin's disease.

MATERIALS AND METHODS

I. Isolation of a novel cDNA clone

A. Analysis of transformed cell lines; development of the differential screening protocol

All tissue culture cell lines were maintained in RPMI 1640 (Hazelton, Lenexa, KA) supplemented with 10% fetal calf serum (Hazelton, Lenexa, KA), 50 U/ml penicillin (Sigma, St. Louis, MO), 50 μg/ml streptomycin (Sigma, St. Louis, MO) and 2 mM L-Glutamine (Sigma, St. Louis, MO). The cells were stored in a humidified chamber at 37°C with 5% CO_2. The Hodgkin's-disease cell line L428 was originally described by Schaadt and collegues (Schaadt *et al.* 1979; Schaadt *et al.*, 1980), and was obtained from Dr. R. Fisher (Department of Hematology/Oncology, Loyola University - Chicago, Maywood, IL). The African Burkitt's lymphoma cell lines Jiyoye and P3HR-1 were provided by Dr. B. Sugden (Department of Oncology, University of Wisconsin - Madison, Madison, WI).

B. Differential screening of the Hodgkin's-disease cDNA library

1. L428 cDNA library

Highly purified poly A^+ RNA was isolated from L428 cells and a cDNA library was prepared by Stratagene (La Jolla, CA). The library was packaged into the Lambda ZAP^{TM} vector. The poly A^+ RNA used to synthesize the cDNA library was prepared as described in section IV.C.1 of the Materials and Methods.

2. cDNA probes

In order to achieve the sensitivity required for detection of genes expressed in low abundance, it was necessary to produce the cDNA molecules that have incorporated a high level of radiolabeled nucleotide. The procedure that I have developed to do so was a combined protocol derived from methods previously described (Gerard, 1988) and from personal communications with several laboratories that are currently using this procedure. Whole cell poly A^+ RNA was isolated from the Jiyoye and P3HR-1 cell lines as described in the sections below. The cDNA was synthesized from the RNA according to the following protocol. Twenty micrograms of poly A^+ RNA was denatured in 20 µl of water at 65^{o}C for 10 minutes. The denatured RNA was then added to a tube containing 60 µl of the following solution: 50 mM Tris (pH 8.3), 40 mM potassium chloride, 8 mM magnesium chloride, 5 mM DTT

(dithiothreitol), 500 μM of unlabeled dCTP, dGTP and dTTP, 10 μM of unlabeled dATP (Pharmacia, Piscataway, NJ) 1 μM of [α^{32}P]-dATP (ICN, Irvine, CA; specific activity greater than 3000 Ci/mmol), and 120 units of RNasin (Promega, Madison, WI). The reduced molarity of the unlabeled dATP is critical for the high level of incorporation of the radiolabeled dATP in the synthesized cDNA molecules. Four thousand (4000) units of Mo-MuLV derived reverse transcriptase (BRL, Bethesda, MD) were added and the reaction was incubated at 42^{o}C for three hours. The RNA template was hydrolyzed by the addition of 120 μl of a 150 mM solution of NaOH (to a final pH = 10.5) and incubating at 70^{o}C for 20 minutes. The solution was then neutralized by the addition of 20 μl of 1 N HCl, extracted twice with phenol, twice with phenol/chloroform, twice with chloroform, and once with ether. The cDNA was then precipitated with ethanol using 0.2 M NaCl and two volumes of ethanol at -85^{o}C for at least one hour and pelleted by centrifugation. The dried pellet was resuspended in 30 μl of TE (pH 7.7), and purified from non-incorporated nucleotides by G-50 Sephadex column chromatography. Determination of peak fractions containing the cDNA was done with a hand held Geiger counter. I typically obtain probes with a specific activity of greater than 10^{9} cpm/μg.

3. Library screening

The cDNA probes prepared above were used to screen the L428 cDNA library packaged in Lambda Zap^{TM} (Stratagene, La Jolla, CA). Standard screening procedures were utilized (Sambrook *et al.*, 1989). Screening protocols were designed so that a 150 mM diameter petri dish would contain 2000 pfu when screening with cDNA probes synthesized from both Jiyoye and P3HR-1 cell mRNA. A 400 µl sample of a suspension of BB4 bacteria, grown to an $O.D._{600}$ = 0.5, were incubated with 600 µl of the Lambda Zap^{TM} library diluted in SM (50 mM Tris HCl [pH = 7.5], 100 mM NaCl, 10 mM $MgSO_4$, 0.01% gelatin) buffer to yield the pfu as described above. The mixture was incubated at 37^{o}C for 15 minutes. To this mixture was then added 8 ml of 0.7% top agarose made in NZYM (10 g/l casein amino acids, 5 g/yeast extract, 10 g/l NaCl, 10 mM $MgSO_4$) media that was previously sterilized and cooled to a temperature of 40-45^{o}C. The tube containing the suspension was quickly inverted several times to evenly suspend the bacteria and poured directly onto a 1.5% NZYM agar plate. Once solidified, the plates were incubated at 37^{o}C until the desired size of plaques developed (approximately five to seven hours), and chilled at 4^{o}C for one hour to harden the top agarose. Duplicate nitrocellulose filters were laid on the plates. The first filter was left on the plate for two minutes, and the second filter was left on

the plate for seven minutes. Orientation of the filters was achieved by piercing through both nitrocellulose and agarose with an 18 gauge needle in an asymmetric pattern. Filters were then incubated in a solution of 1.5 M NaCl and 0.5 M NaOH for two minutes to denature the DNA, neutralized by incubating in a solution of 1.5 M NaCl and 0.5 M Tris (pH 8.0) for five minutes, then washed in 0.2 M Tris (pH 7.5) containing 2X SSC (0.3 M NaCl and 0.03 M sodium citrate) for 30 seconds and dried completely. They were then baked under vacuum at 85^{o}C for two hours. The filters were incubated in prehybridization buffer containing 50% formamide, 1X PIPES buffer, 0.5% SDS, and 0.5 mg/ml denatured salmon sperm DNA in sealed plastic bags for at least one hour at 42^{o}C. The buffer was then removed and replaced with 5 ml of fresh prehybridization buffer and the radiolabeled cDNA probes were added to a final concentration of 10^{7} cpm/ml. The filters were hybridized with the probe at 42^{o}C for 12-24 hours with shaking and then washed according to the following procedure that I have empirically developed to obtain maximum signal to noise ratio: twice in buffer containing 2X SSC, 0.1% SDS at room temperature for 10 minutes, twice in the same buffer at 65^{o}C for 20 minutes, and once in buffer containing 0.1X SSC, 0.1% SDS at 65^{o}C for 10 minutes. Washed filters were then exposed to X-ray film

with intensifying screens at -85°C. Hybridizing plaques were isolated and rescreened to purify specific clones.

C. Characterization of cDNA clones

1. Rescue of cDNA clones

The cDNA inserts from the lambda Zap clones were rescued into the plasmid pBluescript™ following the procedure recommended by Stratagene. Bacteria infected with a lambda clone were incubated with the helper phage R408 provided by Stratagene during which time the cDNA insert was automatically excised and rescued into an *Eco*R I restriction site in pBluescript. The rescued insert in pBluescript was transformed into XL1-Blue cells. Plasmid DNA was prepared by standard procedures (Sambrook, *et al.*, 1989). The plasmid can be used directly to produce capped transcripts suitable for in vitro translation (see below) and for sequencing as described below. The size of each cDNA insert ligated into the plasmid vector, the plasmid DNA was digested with *Eco*R I, separated by agarose gel electrophoresis, stained with ethidium bromide, and detected using a UV transilluminator.

2. Nucleotide sequencing and analysis

Complementary DNA (cDNA) inserts were sequenced in both directions by dideoxy (Sanger) sequencing (Sanger *et al.*, 1977; Sanger *et al.*, 1980) using a US Biochemical Sequenase™ (Cleveland, OH) kit. Following instructions

provided with the kit, 3 μg of double stranded plasmid DNA was denatured in 0.2 N NaOH for five minutes, by the addition of NH_4AC to 0.8 M, and precipitated with two volumes of absolute ethanol. The DNA was pelleted, washed with 70% ethanol, pelleted again, and briefly dried under vacuum.

The oligomer used to prime the elongation reaction was annealed to the denatured plasmid by resuspending the dried DNA in 7 μl water, 2 μl of 5X Sequenase reaction buffer (200 mM Tris-HCl [pH 7.5], 100 mM $MgCl_2$, and 250 mM NaCl] and 1 μl of primer (0.5 pmol/μl). The mix was incubated at 37°C for 15 minutes. The elongation reaction was initiated by adding 2 μl labeling buffer (7.5 μM of each dCTP, dGTP, and dTTP), 1 μl of 0.1 M DTT, 0.5 μl [α^{35}S]-labeled dATP (New England Nuclear, Boston, MA), and 2 μl of diluted Sequenase™ (1 unit/μl in 10 mM Tris-HCl [pH 7.5], 5 mM DTT, and 0.5 mg/ml BSA). The reaction was divided into four tubes (labeled A, C, G, and T respectively) incubated at room temperature for five minutes. Termination was accomplished by adding 2.5 μl of the appropriate ddNTP mix (ddATP, ddCTP, ddGTP, or ddTTP) into the respective tube and incubating for five minutes at 37°C. The sequencing reaction was stopped by the addition of 4 μl of stop solution (95% formamide, 20 mM EDTA, 0.05% bromophenol blue and 0.05% xylene cyanol FF).

Two microliters from each reaction was loaded per well (formed using a sharkstooth comb) on an 8% acrylamide-urea sequencing gel. Electrophoresis was carried out in Tris-boric acid buffer (TBE) at approximately 60 watts constant power (sufficient to maintain the gel at a constant temperature of 50^{o}C). Following electrophoresis, gels were fixed in a solution of 5% methanol and 5% acetic acid for 15 minutes, during which time the urea was removed. The gels were then dried (30 minutes at 80^{o}C) under vacuum using a Hoffer Slab Gel dryer (San Francisco, CA) and autoradiographed for 12 to 24 hours. Each reaction was sufficient to resolve 250 to 300 base pairs of sequence from the primer. Sequence data from both stands of multiple clones was obtained to ensure accuracy. Primers specific for ELAG sequences were made by the Macromolecular Analysis Facility at Loyola University Medical School.

3. Computer analysis - GCG

The sequences were compared to the GenBank/EMBL genetic sequence data bank using the GCG (Genetics Computer Group; University of Wisconsin - Madison) software maintained in the Loyola University department of Academic Computing.

D. Confirmation of the 5' terminus

1. Rescreening the library

To isolate insert cDNAs which were to be used as probes in Northern blot and other hybridization analyses, plasmid DNA was digested with *Eco*R I and DNA separated on a 1% low temperature melting point agarose gel. A 340-bp *Eco*R I fragment (this fragment corresponds to ELAG 1 cDNA sequences between positions 61 and 401; see Figures 4 and 20a) stained with ethidium bromide was localized using a hand held long wave ultraviolet light source and excised from the gel. The gel was then cut into 3 mm squares and placed into preweighed microfuge tubes, diluted with water to a concentration of 0.3 g/ml, boiled for seven minutes and stored at -20°C. This DNA was then radiolabeled with [α^{32}P]-dATP (ICN, Irvine, CA) using the Prime-a-Gene™ labeling kit (Stratagene, La Jolla, CA) in which DNA was replicated with Klenow polymerase following initiation with random primers. Radiolabeled inserts were then separated from non-incorporated nucleotides by G-50 Sephadex chromatography.

2. 5' RACE

Reverse transcription of first strand cDNA was initiated with an ELAG sequence specific primer reverse/complementary to the coding strand (5'-CATT-TTCAAAATCTAGCCAGGC-3'; this sequence corresponds to "primer 1" illustrated in Figure 5 and is reverse/

complementary to sequence positions 657 through 678 shown in Figure 4)). In a standard microfuge tube, 20 ng of poly A^+ RNA was denatured in the presence of 2 pmol of primer 1 at 70°C in a volume of 14 µl for 10 minutes. The denatured RNA/primer mix was immediately chilled on ice for one minute then centrifuged for 20 seconds to collect the condensation. Preparation of the reaction mixture was completed by adding 2 µl of 10x synthesis buffer [200 mM Tris-HCl (pH 8.4), 500 mM KCl, 25 mM $MgCl_2$, 1 mg/ml BSA], 2 µl 0.1 mM DTT, 1 µl 10 mM dNTP mix [10 mM each dATP, dCTP, dGTP, dTTP], and 1 µl of SuperScript™ reverse transcriptase (200 units/µl). The reaction was incubated at 42°C for 30 minutes then stopped by warming to 55°C for five minutes. The template RNA was degraded by the addition of 1 µl *E. coli* RNase H (2 units/µl) and incubating at 55°C for an additional 10 minutes.

The first strand cDNA was separated from the salts, unincorporated nucleotides and primers of the reverse transcription reaction using the GlassMAX™ spin cartridge supplied with the kit. To the 21 µl reverse transcription reaction, 95 µl of binding solution [6 M NaI] were added and the contents transferred to a GlassMAX™ spin column and centrifuged for 20 seconds at 13,000 x g. The cartridge was washed three times with 400 µl of ice cold wash buffer and washed once with 400 µl of ice cold 70% ethanol. The cDNA was eluted by adding 50 µl DEPC

(diethyl pyrocarbonate) treated water (pre-warmed to 65°C) and by centrifugation at 13,000 x g for 20 seconds.

The addition of a poly(dC) tail was essential for the PCR amplification of the cDNA products. In a sterile microfuge tube, 16 µl of purified cDNA were heated for 10 minutes at 70°C, chilled on ice for one minute and centrifuged to collect the condensation. The tailing reaction components were added which include 1 µl 10x synthesis buffer (200 mM Tris-HCl [pH 8.4], 500 mM KCl, 25 mM $MgCl_2$, 1 mg/ml BSA), 2 µl dCTP (2 mM), and 1 µl terminal deoxynucleotidyl transferase (TdT; 10 units/µl). The reaction was incubated for 10 minutes at 37°C. The TdT was heat inactivated at 70°C for 10 minutes and the reaction mixture placed on ice.

The targeted cDNAs were amplified in a reaction containing 5 µl tailed cDNA, 30.5 µl DEPC-treated water, 5 µl 10x synthesis buffer (200 mM Tris-HCl [pH 8.4], 500 mM KCl, 25 mM $MgCl_2$, 1 mg/ml BSA), 1 µl 10 mM dNTP mix (10 mM each dATP, dCTP, dGTP, dTTP), 1 µl of a modified ELAG-specific nested primer (10 µM; 5'-CAUCAUCAUCAUGGAAGTGTTTC-TGATGGTAG-3'; this sequence corresponds to "primer 2" in Figure 5 and is reverse/complementary to sequence positions 315 through 334 shown in Figure 4) and 2 µl of Anchor Primer (5'-CUACUACUAGGCCACGCGTCGACTAGTACGGGIIGGGII-GGGIIG-3'). Four CAU triplet repeats were engineered onto the 5' end of the ELAG specific primer to allow for rapid

cloning into the pAMP-1^{TM} plasmid vector. The reaction components were overlaid with oil and heated to 95^{o}C for five minutes. Prior to starting the PCR cycles, 5 µl of diluted Taq polymerase (0.4 units/µl, Perkin-Elmer Cetus) were added to the reaction mixture below the oil. The cDNA was amplified for 40 cycles (94^{o}C for 30 seconds, 55^{o}C for 30 seconds, 72^{o}C for 30 seconds).

3. Primer extension

Characterization of the transcriptional start site by primer extension was done following the procedure previously described (Makino *et al.*, 1988). Briefly, 2 µg of poly A^{+} enriched RNA was denatured in 8 µl of DEPC treated water containing 10 µM methyl mercury for 10 minutes. The cDNA was reverse transcribed in a 50 µl reaction containing the denatured RNA, 10 µl of 5x cDNA synthesis buffer (500 mM Tris [pH 8.3], 200 mM KCl, 50 mM $MgCl_2$), 10 µl of dNTP mix (25 mM each dATP, dCTP, dGTP, and dTTP; Pharmacia, Piscataway, NJ) and 1 µl of AMV reverse transcriptase (2 units/µl; Seikagaku, Rockville, MD), 28 mM 2-mercaptoethanol and 1 pmol of 5' end-labeled primer (5'-GTTTTGCATCTATACCTCTCC-3'; this primer is reverse/complementary to the sequence positions 146 through 126 shown in Figure 4). The reaction was incubated for one hour at 42^{o}C, phenol/chloroform extracted, ethanol precipitated and resolved on a standard sequencing gel.

The primer was end-labeled following standard procedures (Sambrook *et al.*, 1989) using [γ-^{32}P]-dATP (6000 Ci/mmol; New England Nuclear, Boston, MA).

II. Analysis of ELAG transcription by reverse transcription/polymerase chain reaction

A. Peripheral blood mononuclear cells

All of the cell lines and normal peripheral blood lymphocytes were cultured in RPMI 1640 (Hazelton, Lenexa, KA) supplemented with 10% fetal calf serum (Sigma, St. Louis, MO), 500 units/ml penicillin G (Sigma, St. Louis, MO) and 50 µg/ml streptomycin sulfate (Sigma, St. Louis, MO). Cultures were maintained in a humidified incubator at 37°C with 5% CO_2 in 25 cm^2 or 75 cm^2 tissue culture flasks or tissue culture plates.

1. Isolation

Venous heparinized blood was collected from normal donors, diluted 1:1 with RPMI 1640 without serum, and 40 ml layered onto 10 ml of Histopaque™ (Sigma, St. Louis, MO) separation medium. The tube was centrifuged at 600 x g and the layer containing the white blood cells collected and washed in RPMI 1640. Cell pellets were then either frozen at -85°C for RNA extraction from resting cells, or cultured in vitro as described below.

2. Activation

Mononuclear peripheral blood cells were aliquoted in 24 well tissue culture cluster plates (Costar, Cambridge, MA) at a concentration of 1 x 10^6 cells/ml RPMI 1640 supplemented with 10% heat inactivated fetal calf serum and incubated at 37°C with 5% CO_2 with either 10 μg/ml of phytohemagglutinin (PHA; Sigma, St. Louis, MO) or 50 μg/ml anti-human Leu-4 monoclonal antibody (Becton-Dickenson, San Jose, CA) which specifically binds to the CD3 molecule (also referred to as anti-CD3 in this paper). These concentrations were previously determined to affect the optimal cellular proliferation as measured by [^{3}H]-thymidine uptake analysis.

Cells from the Jurkat human T cell leukemic line were incubated with the tumor promoter phorbol 12-myristate 13-acetate (PMA; Sigma, St. Louis, MO) at a standard concentration of 10 ng/ml, PHA (Sigma; St. Louis, MO) at 10 μg/ml or both at the concentrations indicated. Cells were harvest at four hours or 24 hours following stimulation of the cells, washed in phosphate buffered saline, and used for RNA isolation.

B. Reverse transcription/Polymerase chain reaction (RT/PCR)

1. RNA preparation

For all procedures involving RNA, steps were taken to minimize degradation of RNA by treating all solutions with DEPC followed by autoclaving and by baking all glassware at 250°C overnight.

Poly A^+ RNA used in the RT/PCR studies was isolated from cell pellets using the QuickPrep™ Micro mRNA purification kit from Pharmacia (Piscataway, NJ) following the suggested protocol. Briefly, a cell pellet containing 10^7 cells was lysed in 400 µl of Extraction buffer (aqueous solution containing guanidinium thiocyanate and N-lauroylsarcosine), and vortexed vigorously for one minute. The cell lysate was diluted in 800 µl of Elution buffer (10 mM Tris-HCl [pH 7.4], 1 mM EDTA), and vortexed for an additional minute. The lysate was cleared by centrifugation at 13,000 x g for one minute and the cleared homogenate was added to an oligo(dT)-cellulose pellet prepared from 1 ml of slurry. The homogenate and oligo(dT) were mixed thoroughly for three minutes to ensure efficient binding of RNA to the oligo(dT). The oligo(dT) was pelleted, washed five times with high salt buffer (10 mM Tris-HCl [pH 7.4], 1 mM EDTA, 0.5 M NaCl), twice with a low salt buffer (10 mM Tris-HCl [pH 7.4], 1 mM EDTA, 0.1 M NaCl), and transferred to a MicroSpin™

column. The pellet was washed an additional two times in the column. The RNA was eluted by adding 70 µl of 65°C elution buffer and centrifugation for five seconds at 13,000 x g. The elution was repeated two more times to ensure efficient removal of RNA. RNA was stored at -85°C.

2. Reverse transcription - cDNA synthesis

Complementary DNA (cDNA) was synthesized from cell lines as well as resting and activated PBMCs using modifications of standard protocols. Twenty micrograms of poly A^+ RNA was denatured in 11 µl of water and 1 µl of random hexamers (1 µg/µl; Pharmacia, Piscataway, NJ) at 70°C for 10 minutes, then chilled on ice for one minute and centrifuged briefly to collect the condensation. Reverse transcription was initiated by adding 4 µl of 5x reaction buffer (500 mM Tris [pH 8.3], 200 mM KCl, 50 mM $MgCl_2$), 2 µl of dNTP mix (25 mM each dATP, dCTP, dGTP, and dTTP; Pharmacia, Piscataway, NJ) and 1 µl of AMV reverse transcriptase (2 units/µl; Seikagaku, Rockville, MD) and incubated at 42°C for 30 minutes. The reaction was terminated by heating to 70°C for five minutes. A control reaction was prepared for each RNA sample tested by assembling a standard reaction mixture and replacing reverse transcriptase with water.

3. Polymerase chain reaction

Polymerase chain reaction (PCR) was done following standard protocols with commercially available reagents.

To enhance the efficiency and specificity of the reaction, a hot start was used. In the hot start procedure, each reaction was assembled in a 0.5 ml PCR tube and contains 10 µl of 10x synthesis buffer (100 mM Tris-HCl [pH 9.0 at 25°C, 500 mM KCl, 1% triton X-100), 16 µl of $MgCl_2$, 8 µl of dNTP mix (10 mM each dATP, dCTP, dGTP, and dTTP; Pharmacia, Piscataway, NJ), 1 µl of primer 1 (10 µM; 5'-TCCCATCAAGCAGAGAAGTAG-3'), 1 µl of primer 2 (10 µM; 5'-CCAGTTGGAAAGTGTTTCTGATG-3'). The efficiency of each reaction is highly dependent of the concentration of Mg^{++}, which I empirically determined to be 4 mM for the reactions done in this lab. Each reaction was covered with an Ampliwax™ pellet (Perkin-Elmer Cetus, Norwalk, CT), heated to 80°C for three minutes to melt the wax, then cooled to room temperature to solidify the wax. Each reaction mix was covered with 56 µl of water and 5 µl diluted Taq polymerase (Promega, Madison, WI) and 5 µl of cDNA. As a control to determine that cDNA was made from each RNA sample, 5 µl of cDNA was amplified using primers (5'-GCCTGCCGTGTGAACCACGTGAC-3' and 5'-TACCTGTGGAGCAACCTGC-TCAGA-3') which amplify a 277-bp segment of the Beta-2-microglobulin gene (Lu *et al.*, 1992). All amplification programs were initiated with a five minute incubation at 95°C to fully denature the cDNA template and primers. The standard program used for the RT/PCR experiments described in this study was a three temperature cycle (95°C for 30

seconds, 55°C for 30 seconds, and 72°C for 30 seconds). The program concludes with a 10 minute incubation at 72°C to ensure that all reactions are completed. Each reaction was supplemented with 1 μCi [α^{32}P]-dATP (3000 Ci/mmol; New England Nuclear, Boston, MA) to label the amplified fragments of DNA. Products were separated by electrophoresis on a 2% agarose gel, dried, and autoradiographed using an enhancing screen at -85°C.

The linear range of amplification for the 277-bp Beta-2-microglobulin segment and the 326-bp ELAG segment from L428 cDNA were empirically determined following an established protocol (Higuchi, 1990). I determined that the linear range of amplification for Beta-2-microglobulin is between 22 and 30 cycles, while the linear range of amplification of ELAG expressed in L428 cells is between 22 and 32 cycles. All PCR reactions initiated to specifically amplify a segment of the Beta-2-microglobulin gene were amplified for 30 cycles. However, it was necessary to amplify ELAG products from activated PBMCs for 36 cycles because of the relatively low level of ELAG expression.

4. Southern blot hybridization.

PCR amplification of the ELAG specific segment was carried out as described above except that the reaction was done in the absence of radiolabeled dATP. PCR products were separated by agarose gel electrophoresis

using standard methodology (Sambrook *et al.*, 1989). Following electrophoresis, the gel was denatured with 50 mM NaOH for 30 minutes, neutralized in 0.1 M Tris-HCl (pH 7.0) for 30 minutes and the DNA transferred to GeneScreen Plus^{TM} (NEN, Boston, MA) using a vacuum transfer apparatus and 10X SSC. The blots were denatured for 60 seconds in 50 mM NaOH, neutralized, and dried. The membrane was then incubated in pre-hybridization buffer containing 50% formamide, 0.5 M NaCl, 10% dextran sulfate, and 20 μg per milliliter denatured salmon sperm DNA for at least one hour at 42^{o}C. The buffer was changed and the blots hybridized with radiolabeled probes prepared from a 340-bp *Eco*R fragment of ELAG cDNA. Blots were washed in several changes of 2X SSC and 0.1% SDS at room temperature, and at 65^{o}C in 0.1X SSC and 0.1% SDS. Autoradiography was done to detect ELAG hybridizing genomic fragments.

III. Characterization of the putative ELAG-encoded protein

A. Computer analysis

Analysis of open reading frames and structural motifs was done using the MacVector software (IBI, New Haven, CT).

B. In vitro translation

The ELAG transcripts were synthesized from linearized plasmid containing cDNA which encodes the ELAG open reading frame. The cDNA inserts were cloned into the pGEM^{TM} (Promega, Madison, WI) vector and sequenced to determine that transcription from the T7 RNA polymerase promoter would produce the sense RNA strand. The plasmid was linearized by standard restriction digest using Sal I which will cleave the plasmid in the polylinker region on the 3' end of the insert. Digested plasmid was analyzed by electrophoresis on 1% agarose gel to verify the absence of detectable non-linearized DNA.

In an RNAse-free microfuge tube, 1 μg of linearized DNA template was combined with 2 μl of 2.5 mM m7G(5')ppp(5')G cap analog (New England Biolabs, Beverly, MA), 4 μl of 5x buffer (200 mM Tris-HCl [pH 7.9], 30 mM $MgCl_2$, 10 mM spermidine, and 50 mM NaCl), 2 μl of 0.1 M DTT, 3 μl of a nucleotide mix (which was 2 μM of ATP, CTP, and UTP), 1 μl of 0.25 mM GTP, 0.5 μl RNasin, and 1 μl of T7 RNA polymerase (20 units/μl; Promega, Madison, WI) and incubated for one hour at 37^{o}C. The concentration of GTP was critical to efficient capping and was empirically determined in our laboratory. The amount of transcript was estimated by electrophoresis in a 1% formaldehyde containing agarose gel and staining with ethidium bromide.

The in vitro translation was performed using a wheat germ translation system (Promega, Madison, WI) following the manufacturer's suggested protocol. A 50 µl reaction was assembled in an RNAse-free microfuge tube. Five hundred nanograms of RNA was denatured at 70°C for 10 minutes then immediately chilled on ice. The components of the translation reaction were added which include 25 µl of wheat germ extract (in a buffer containing 107.5 mM potassium acetate, 4.2 mM magnesium acetate, 5 mM 2-mercaptoethanol, 10 mM DTT, 0.1 mg/ml tRNA, 1 mM spermidine, HEPES (pH 7.5; the concentration of HEPES buffer in the lysate is not provided by the company), 4 µl of a 1 mM amino acid mixture minus cysteine, 2.5 µl [^{35}S]-cysteine (1200 Ci/mmole; New England Nuclear, Boston, MA), 0.5 µl RNasin (40 units/µl), and 15.5 µl DEPC-treated water. The reaction was incubated at 25°C for one hour and terminated by adding 50 µl of 2X Laemli sample buffer.

C. Mutagenesis

To test the ability of CUG to serve as the ELAG translation initiation site, a CUG to TTA mutation in the translational start site was created using a primer mediated mutagenesis (Higuchi *et al.*, 1988; Higuchi, 1990). The procedure is illustrated in Figure 14. Primers B (5'-GGGTGAGCAGAGTTACCAAGAGCAAAA-3') and C (5'-TTTTGCTCTTGGTAACTCTGCTCACCC-3') are complementary to each

other; identical to the double stranded DNA between positions 43 to 67 except that the CTG at position 55 was changed to TTA on primer B. Likewise, the CAG (reverse complimentary to CTG) was changed to TAA (reverse complimentary to TTA) on primer C. In the first two PCR reactions primers A and B were used in a single reaction while primers C and D were used in the other reaction. The expected products would overlap and contain the substitutions that allow for the CTG to TTA change. The PCR products were purified to remove unincorporated nucleotides and primers by gel purification and elution with the Magic PCR Prep^{TM} system (Promega, Madison, WI). The purification was done by electrophoresis in a 1% low melting temperature gel. The ethidium bromide stained bands were excised, melted at 70^{o}C for five minutes, then diluted in 1 ml of PCR Prep resin (resin containing 7 M guanidinium thiocyanate, and glass beads.). The slurry was collected in a column by vacuum and washed with 70% isopropyl alcohol. The DNA was eluted with 5 µl of water and centrifuged at 16,000 x g for 30 seconds. The purified PCR products were introduced into a single PCR (steps 2 and 3) reaction containing the outside primers; A and D. The mutated cDNA was purified again by gel electrophoresis and separation from the agarose using Magic PCR Prep^{TM} (Promega, Madison, WI). Purified cDNA was subcloned into the pGEM^{TM} PCR cloning vector (Promega,

Madison, WI) in a ligation reaction containing 10 ng of PCR product, 1 µl of vector (10 ng/µl) 1 µl of 10x ligase buffer (300 mM Tris-HCl [pH 7.5], 100 mM $MgCl_2$, 100 mM DTT, 10 mM ATP), 1 µl of T4 DNA ligase, and water to a final volume of 10 µl. The ligation reaction was incubated at 14°C overnight, then used to transform competent E. coli cells by standard protocol. The subcloned cDNA insert was sequenced to confirm that the intended bases were mutated.

IV. Characterization of the transformation potential of ELAG

A. 3' RACE

In an effort to compare the sequences of the ELAG transcripts expressed in normal activated cells versus the transformed Hodgkin's-disease cells, cDNA clones were isolated using 3' RACE (Rapid Amplification of cDNA Ends). Reverse transcription of the first strand cDNA was initiated with a modified poly(dT) primer designated as the Adapter Primer (5'-GGCCACGCGTCGACTAGTAC$(T)_{17}$-3'). The Adapter primer (1 µl of a 10 mM solution) was added to 20 ng of poly A^+ RNA in a total volume of 14 µl and denatured at 70°C for 10 minutes. The mixture was chilled on ice for one minute and centrifuged to collect the

condensation. The remaining reaction components were added which include 2 µl of 10x synthesis buffer, [200 mM Tris-HCl (pH 8.4), 500 mM KCl, 25 mM $MgCl_2$, 1 mg/ml BSA], 2 µl 0.1 mM DTT, 1 µl 10 mM dNTP mix [10 mM each dATP, dCTP, dGTP, dTTP], and 1 µl of SuperScript™ reverse transcriptase (200 units/µl). The RNA template was degraded by adding 1 µl of E. coli RNAse H (2 units/µl) and incubated at 42°C for an additional 10 minutes. The condensation was collected by quick centrifugation.

The target cDNA was amplified in a reaction mixture containing 5 µl of first strand cDNA, 39.5 µl DEPC-treated water, 5 µl 10x synthesis buffer (200 mM Tris-HCl [pH 8.4], 500 mM KCl, 25 mM $MgCl_2$, 1 mg/ml BSA), 1 µl of 10 mM dNTP mix 1 µl 10 mM dNTP mix (10 mM each dATP, dCTP, dGTP, dTTP), 1 µl of an ELAG-specific primer (5'-CAUCAUCAUCAUG-CAAAAAGAAACCATTCATATC-3'; primer 1 illustrated in Figure 17 and contains ELAG sequences identical to sequence positions 64 through 85 shown in Figure 4) prepared as a 10 µM solution, and 2 µl of the Universal Amplification Primer (10 µM, 5'-CUACUACUACUAGGCCACGCGTCGACTAGTAC-3', BRL, Bethesda, MD). Four CAU triplet repeats were engineered onto the 5' end of the ELAG-specific primer to allow for rapid cloning into the pAMP-1™ plasmid vector. The reaction components were overlaid with oil and heated to 95°C for five minutes. Prior to starting the PCR cycles, 5 µl of diluted Taq polymerase (0.4 units/µl,

Perkin-Elmer Cetus, Norwalk, CT) were added to the reaction mixture below the oil. The cDNA was amplified for 40 cycles (94°C 30 seconds, 55°C for 30 seconds, 72°C for 30 seconds).

The RACE products were cloned into the pAMP-1 vector using the CloneAmp™ system (Gibco BRL, Bethesda, MD). The plasmid pAMP-1 (1 µl containing approximately 50 ng) was combined with 8 µl of amplified product from either a 5' or a 3' RACE reaction in a sterile microfuge tube. To the mixture, 1 µl of uracil DNA glycosylase (1 unit/µl) was added and incubated for 30 minutes at 37°C. Following the subcloning procedure, 1 µl of the ligation mixture was used to transform competent bacteria. Clones containing the ELAG DNA were identified by hybridization with an ELAG probe, analyzed by restriction digest and gel electrophoresis.

B. 5' RACE

The 5' terminal sequences of each clone isolated were confirmed by 5' RACE using the same procedure as described in section I.D.2 of the Materials and Methods. The primer used in this procedure to initiate reverse transcription was 5'-TCCTCCAATGCAAGATCC-3' which is reverse complementary to sequence positions 488 through 505 as shown in Figure 18. The ELAG primer used to amplify cDNA products was 5'-CAUCAUCAUCAUGAATCACACATTGCATCGAG-3' which

contains sequences that are reverse/complementary to sequence positions 462 through 481 as shown in Figure 18.

C. Northern blot analysis

1. Isolation of RNA

Whole cell RNA was extracted using modifications of the procedure originally described previously (Chirgwin *et al.*, 1979). Briefly, cell pellets were lysed in buffer containing 4 M guanidinium thiocyanate (Fluka, Ronkonkoma, NY), 0.5% sodium N-lauroylsarcosine, 25 mM sodium citrate, and 0.1 M 2-mercaptoethanol, and lysate was then layered over a 5.7 M CsCl (Schwartz-Mann, Cleveland, OH) cushion. The RNA was pelleted through the CsCl at 30,000 RPM in a SW41 Beckman rotor for 24 hours. The RNA pellet was dried and washed by ethanol precipitation. Concentration of the RNA was estimated by spectrophotometric analysis. Definitive analysis of the quality of RNA was determined by separating the RNA on formaldehyde containing agarose gels and stained with ethidium bromide to detect the 28S and 18S ribosomal bands.

Poly A^+ RNA was enriched from RNA preparations by oligo(dT) affinity chromatography using procedures as adapted in our laboratory. The whole RNA was resuspended in 2 ml loading buffer containing 10 mM Tris (pH 7.0), 10 mM EDTA, 0.5% SDS, and 0.5 M NaCl and applied to a oligo(dT) cellulose (BRL, Bethesda, MD) column

equilibrated in the same buffer. The solution containing RNA was added to the column, the effluent collected and added back to the column. The effluent following this step was discarded. The column resin was washed extensively with the loading buffer. The bound mRNA fraction was eluted with buffer containing 10 mM Tris (pH 7.0), 0.05% SDS and 1 mM EDTA, peak fractions pooled, and the mRNA precipitated with 0.2 M NaCl and ethanol at -85°C for one hour. The oligo(dT) resin was regenerated with 0.2 N NaOH and washed in loading buffer. The mRNA was collected by centrifugation, dried, resuspended in loading buffer, and chromatography was repeated as before. Enrichment of mRNA was assessed by formaldehyde containing agarose gel electrophoresis of whole RNA, the effluent ribosomal fraction, and the poly A^+ enriched RNA fraction. RNA was detected by ethidium bromide staining and the use of a UV transilluminator. I was typically able to achieve greater than 90% enrichment of poly A^+ RNA and used this procedure to isolate RNA for the production of the L428 cDNA library.

2. Formaldehyde gel electrophoresis

The quality of RNA preparations was determined by electrophoresis through formaldehyde containing agarose gels following protocols outlined previously (Sambrook *et al.*, 1989). Briefly, a 100 ml agarose (1% w/v) gel was prepared by melting 1 g of agarose in 74 ml of water. The

agarose was allowed to cool to 60°C, then 10 ml of 10X MOPS buffer (0.2 M MOPS [pH 7.0], 80 mM sodium acetate, and 10 mM EDTA [pH 8.0]), and 16 ml formaldehyde (final concentration was 2.2 M) were added. Gels were run at 100 volts in a 1X MOPS buffer with constant recirculation to maintain pH. The RNA was denatured in 50% formamide, 2.2 M formaldehyde, and 1X MOPS for 10 minutes at 65°C. After a denaturing step, 10X loading buffer (50% glycerol, 1 mM EDTA [pH 8.0], 0.25% bromophenol blue, and 0.25% xylene cyanol FF) was added to each sample.

3. Transfer and process

The procedure used in our laboratory for northern blot analysis was a modification of standard procedures (Sambrook et al., 1989). Briefly, poly A^+ RNA was transferred from the formaldehyde gel onto a piece of GeneScreen Plus™ (NEN, Boston, MA) using a vacuum blot transfer apparatus and 10X SSC. Total transfer was achieved in two hours. To remove formaldehyde and renature the RNA, the membrane was then washed at room temperature in 50 mM NaOH for 15 seconds, and then neutralized in buffer containing 1X SSC and 200 mM Tris (pH 8.0) for 30 seconds. The membrane was incubated in a prehybridization solution containing 1 M NaCl, 1% SDS, and 10% dextran sulfate for one hour at 65°C. Hybridization was done in fresh prehybridization solution supplemented with 50 μg/ml sheared salmon sperm DNA and [^{32}P]-labeled

probe (1 x 10^6 cpm/ml) for 12 to 18 hours at 65°C. Blots were washed twice in buffer containing 2X SSC, 0.1% SDS at room temperature for 10 minutes, twice in the same buffer at 65°C for 20 minutes, and once in buffer containing 0.1X SSC, 0.1% SDS at 65°C for 10 minutes. Washed filters were then exposed to X-ray film with intensifying screens at -85°C.

V. Transcriptional control of ELAG

A. Screening the genomic library

The genomic library prepared from the WI-38 human lung fibroblast cell line was purchased from Stratagene. The library was packaged in the Lambda Fix™ vector. The library was screened following the procedure described in section I.B.3 of Materials and Methods in which a 340-bp *Eco*R I fragment from ELAG 1 was radiolabeled and used as a probe. This fragment (from sequence position 58 to 398 shown in Figure 4) contains most of the open reading frame. Plaque purified clones were amplified and the DNA was harvested by the following method. The KH803 strain of E. coli was infected with the phage at a 200:1 bacteria to phage ratio in a 3 ml culture, and incubated while shaking at 37°C for 20 minutes. The culture was added to 500 ml NZCYM broth in a 2L flask. This large culture was

incubated at 37°C with shaking for approximately 10 hours until complete lysis was observed. NaCl was then dissolved to a final concentration of 1 molar and the culture chilled on ice for one hour. The lysate was cleared by centrifugation at 16,000 x g for 10 minutes at 4°C. Phage were precipitated by adding polyethylene glycol to the supernatant and chilling on ice. The precipitate was pelleted by centrifugation at 16,000 x g for 10 minutes at 4°C. The supernatant was discarded and the pelleted phage particles were resuspended in 10 ml of SM and 7.5 g of cesium chloride was dissolved. The suspension was centrifuged at 150,000g for 24 hours. The phage band was removed from the gradient and dialyzed against a buffer containing 500 mM Tris-HCl (pH 7.7), 25 mM KCl, and 0.5 mM $MgCl_2$. The phage were then lysed by adding EDTA, SDS and Proteinase K (to a final concentration of 50 mM, 0.5% and 100 μg/ml respectively) and incubating at room temperature for 30 minutes. The mixture was phenol/chloroform extracted and ethanol precipitated by adding sodium acetate to a concentration of 300 mM and two volumes of ethanol. The DNA was resuspended in T.E. and digested with the various restriction enzymes. The restriction fragments were separated by electrophoresis in a 1% agarose gel, stained with ethidium bromide and analyzed.

Restriction fragments containing sequences of interest as indicated by hybridization studies were subcloned into pBluescript™ by the following protocol. Restriction enzyme digested DNA was separated on a 1% low melting temperature agarose gel and stained with ethidium bromide. The bands of interest, including the pBluescript™ vector linearized with the appropriate enzyme, were localized using a long wavelength ultraviolet light source, and excised. An individual insert was ligated into vector DNA in a reaction containing 5 µl of water, 1 µl of melted agarose slice containing vector DNA (approximately 50 ng), 4 µl of melted agarose slice containing the intended insert and warming the tube for 10 minutes at 37°C. The reaction mixture was completed by adding 10 µl of a ligase cocktail containing 10 units of T4 DNA ligase, 10 mM DTT, 10 mM ATP and ligase buffer (60 mM Tris-HCl [pH 7.8], 20 mM $MgCl_2$). The reactions were incubated at 14°C overnight.

B. Bacterial strains and transformation

Our laboratory typically transforms E. coli strains XL1-Blue, DH5-α, or JM109 with ligated plasmid DNA. The cells were made competent following a standard protocol. A single colony was picked from an LB plate (LB + 15 µg/ml of tetracycline was used for XL1-Blue) and used to inoculate 5 ml of YA (5% Bacto yeast extract, 2% Bacto

tryptone, 10 mM $MgSO_4$, pH to 7.6 with KOH) and incubate while shaking at 37°C to an $O.D._{550}$ = 0.3. The culture was added to 100 ml YA in a 1 liter flask and continued shaking at 37°C until the culture attained a density of $O.D._{550}$ = 0.48. The culture was then chilled on ice for five minutes and the cells pelleted by centrifugation at 1000 x g, 4°C, for five minutes. The supernatant was removed and the cells were resuspended in 40 ml ice cold Tfb1 (30 mM KAc, 100 mM KCl, 10 mM $CaCl_2$, 50 mM $MnCl_2$, 15% glycerol, pH to 5.8 with acetic acid, sterilized by filtration at 0.22μm). Following a five minute incubation on ice the cells were again pelleted at 1000 x g at 4°C for five minutes. The supernatant was removed and the cells were resuspended in 4 ml ice cold Tfb2 (10 mM MOPS [pH 6.5], 75 mM $CaCl_2$, 10 mM KCl, 15% glycerol, sterilized by filtration at 0.22μm). The cells were incubated on ice for an additional 15 minutes then aliquoted, flash frozen in liquid nitrogen and stored at -85°C. These cells typically have an efficiency of approximately 1 x 10^7 cfu/μg of supercoiled DNA.

Transformation of competent cells was performed by standard procedures. Falcon 2054 tubes were pre-chilled on ice for two minutes and 50 μl of thawed competent cells were added to each tube. Between 1 ng and 100 ng of DNA were added to each aliquot and stored on ice for 30 minutes. The cells heated to 42°C for 90 seconds and

returned to ice for an additional two minutes. The cells were diluted in 950 µl of SOC (2% Bacto tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose) and incubated with shaking at 37°C for one hour. The cells were then plated on LB (1% Bactotryptone, 1% NaCl, 0.5% Bacto Yeast extract) containing agar and supplemented with the appropriate antibiotic.

RESULTS

I. Isolation and sequence of a novel cDNA clone

A. Screening protocol

Based on recent evidence associating Epstein-Barr virus (EBV) infection with the development of Hodgkin's disease (Anagnostopoulos *et al.*, 1989; Weiss *et al.*, 1989; Staal *et al.*, 1989; Libetta *et al.*, 1990), I hypothesized that the oncogenic events responsible for Hodgkin's disease, both EBV associated and non-EBV associated Hodgkin's disease, are mediated by aberrant expression of cellular genes which are also aberrantly expressed in EBV infected non-Hodgkin's lymphoma. My objective therefore, was to isolate cellular genes highly expressed in association with Hodgkin's disease and EBV infected Burkitt's lymphomas in order to begin elucidating the signal transduction pathways responsible for the transformation events in Hodgkin's disease. When I initiated this study, the CD30 gene had been associated with the Hodgkin's disease phenotype and had been shown to be expressed in some EBV infected Burkitt's lymphoma cells. Therefore, I was interested in isolating this gene, or any novel gene potentially involved in the

tumorigenic transformation of cells to the Hodgkin's disease phenotype.

To do so, I developed a screening procedure that would identify genes expressed in an EBV negative Hodgkin's-disease cell and that are potential cellular targets of the EBV encoded nuclear antigen 2 (EBNA 2). EBNA 2 is a viral gene important for inducing cellular immortalization (Cohen *et al.*, 1989; Cohen *et al.*, 1992). The differential screening protocol employed the use of three cell lines; L428, Jiyoye, and P3HR-1. Some characteristics of these cell lines which are relavant to the screening procedure are summarized in Table 1. The L428 cell line is an accepted tissue culture derivative of the Hodgkin's disease/Reed-Sternberg cell (H/RS) (Drexler *et al.*, 1986; Drexler and Minowada, 1992). The cell line was derived from the pleural effusion of a Hodgkin's disease patient (Schaadt *et al.*, 1980). Several cellular proteins commonly associated with Hodgkin's disease are expressed by this cell line including CD30 (Schwab *et al.*, 1982; Stein *et al.*, 1985; Nawrocki *et al.*, 1988) and IL-9 (Gruss *et al.*, 1992). Characterization of EBNA expression shows that the L428 cell line is not infected with EBV (Schaadt *et al.*, 1980). The Jiyoye cell line was established from an African Burkitt's lymphoma (Hinuma and Grace, 1967). The cell is infected with a strain of EBV that encodes EBNA 2. Virus isolated from tissue culture

TABLE 1

CELL LINE	DESCRIPTION	EBV	EBNA 2
L428	Hodgkin's disease	-	-
Jiyoye	African Burkitt's lymphoma	+	+
P3HR-1	African Burkitt's lymphoma	+	-

Characteristics of the cell lines utilized in the differential screening procedure. EBV, Epstein-Barr virus; EBNA-2, Epstein-Barr nuclear antigen 2.

supernatants of Jiyoye cells infects and immortalizes peripheral blood B lymphocytes. The P3HR-1 cell line is a tissue culture derivative of the Jiyoye cell line (Hinuma and Grace, 1967). These cells are infected with a strain of EBV that lacks the gene encoding EBNA 2 due to a spontaneous deletion (Jeang and Hayward, 1983; Bornkamm *et al.*, 1982). Virus isolated from the tissue culture supernatants of P3HR-1 cells can infect peripheral blood B lymphocytes, but the infected cells will fail to become immortalized (Jeang and Hayward, 1983; Bornkamm *et al.*, 1982). Additionally, the Hodgkin's-disease related CD30 is expressed in the Jiyoye cell line but not in the P3HR-1 cell line.

The differential screening procedure used is diagramed in Figure 1. The cDNA library from the EBV negative, Hodgkin's-disease cell line L428 was screened by standard protocols with some modifications as described in the Materials and Methods section. Duplicate nitrocellulose filters were prepared from the library. One of the nitrocellulose filter was hybridized with high specific activity [^{32}P]-labeled cDNA probes from the Jiyoye cell line. The duplicate filter was hybridized with cDNA from the P3HR-1 cell line. Following hybridization and washing, the nitrocellulose discs were autoradiographed. In the hypothetical autoradiographic constellation illustrated in Figure 1, the clone in the

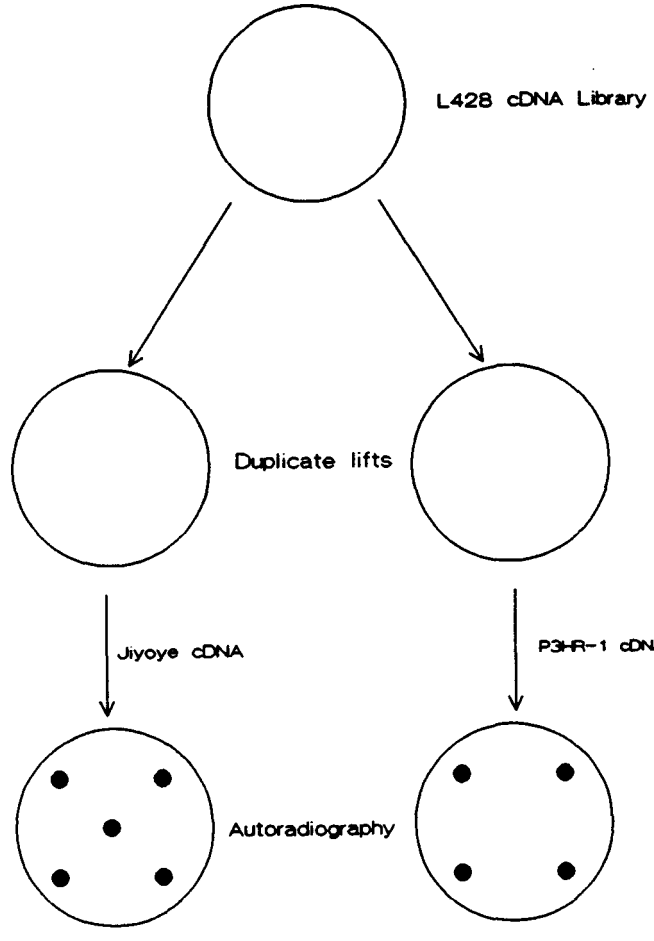

FIGURE 1. Schematic diagram of the differential screening protocol. The cDNA library prepared from the Hodgkin's-disease cell line L428 was differentially screened by preparing duplicate lifts on nitrocellulose filters, and hybridizing one nitrocellulose filter with high specific activity cDNA from the Jiyoye and hybridizing the duplicate filter with cDNA probes from the P3HR-1 cell line. The hypothetical autoradiographs shown in this figure illustrate a clone that specifically hybridizes with the Jiyoye probe and that would be a target for further study.

center of the filter hybridized with Jiyoye cDNA represents a gene expressed in common between Jiyoye and L428 but not in P3HR-1.

Clones isolated by this method were tested to determine if they meet three criteria: 1) I was interested in novel genes. One such gene of interest was the CD30 gene, which at the time of the screening had not been isolated; 2) isolated clones should also be expressed in association with Hodgkin's disease and EBV mediated transformation; 3) finally, I was interested in clones expressed in association with normal lymphoid cell growth, not housekeeping genes, since many transformation associated genes are normally involved in growth and proliferation (Bishop, 1987).

B. Sequence analysis

To satisfy the first criteria described above, clones isolated by the differential screening procedure were partially sequenced. Three clones which were selected following three rounds of differential screening were rescued into the pBluescript™ plasmid. The cDNA inserts were sequenced by the Sanger chain termination method using primers for the T7 RNA polymerase promoter on the plasmid vector. Sequences were compared with the GenBank/EMBL data bases using the GCG computer software.

Partial sequences of two clones isolated by differential screening are shown in Figure 2. A clone designated as H4 was compared to the data base and the best match is shown in Figure 2A. The results show that the sequence between position 3 and 122 were nearly identical to the sequence of the HLA-Cw2 heavy chain gene between positions 4013 and 3840. Only two mismatches were identified (positions 13 and 101 of the H4 clone). Close analysis of the autoradiograph with the actual sequence data from the H4 clone indicates that these mismatches are potentially due to sequencing errors (data not shown). The numerical positions of the HLA clone are shown in descending order. Additionally, the HLA sequences shown in this figure are complementary to published sequences between positions 3840 and 4013 (Parham *et al.*, 1989). These results taken together indicate that the computer generated comparison has aligned the partial sequence of the H4 clone with the 3' terminus of the HLA sequence and therefore, H4 is probably not a novel clone.

A clone designated as F2T3 was compared to the data base and the best match is shown in Figure 2B. The results show that the sequence between position 1 and 88 were nearly identical to the sequence of the mitochondrial encoded cytochrome oxidase III (COIII) gene between positions 9990 and 9903. Four mismatches were identified (positions 43, 44, 51, and 83 of the F2T3 clone). Close

A

```
H4.Txt                    check: 6038      from: 1          to 207          /Reverse
Primate:Hummhcw2b         check: 6881      from: 3809       to: 4023
        Human MHC class I HLA-Cw2 heavy chain gene, complete cds. 6/89
Gaps: 5      Quality: 118.0     Ratio: 0.678     Score: 122     Width: 5     Linits: +/-6

  178 tgtctcaactttatgtgcactgagctgcacacttccttcacttcccctta 129
      |||||| | | | |||   || | ||  ||   | ||   || |  |||
 3840 TGTCTCCA.TCTCTGT...CTCAACTTTACGTGTACTGAGCTGCAACTT. 3884

  128 acctt......ggaaaataagaatctgaatatacatttgttttctcaaat 85
      |||        ||||||||||||||||||||| ||||||||||||||||
 3885 ..CTTCCCTACTGAAAATAAGAATCTGAATATAAATTTGTTTTCTCAAAT 3932

   84 atttgctatgagaggttgatggattaattaaataagtcaattcctggaat 35
      |||||||||||||||||||||||||||||||||||||||||||||||||
 3933 ATTTGCTATGAGAGGTTGATGGATTAATTAAATAAGTCAATTCCTGGAAG 3982

   34 gtgagagagcaaataaagacctgagaaccttc 3
      |||||||||||||||||||| ||||||||||
 3983 TTGAGAGAGCAAATAAAGACC.GAGAACCTTC 4013
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B

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F2T3.Txt                  check: 9755      from: 1          to 88           /Reverse
Organelle:Hummtcg         check: 9132      from: 9902       to: 16569
        Human mitochondrion, complete gonome,. 6/89
Gaps: 2      Quality: 77.6      Ratio: 0.882     Score: 87      Width: 9     Linits: +/-10

   88 TTTGGC.TCGAAGCCGCCGCCTGATACTGGCATTTTGTAAGATGTCCTTT 40
      |||||| |||||||||||||||||||||||||||||| |||||| |||
 9903 TTTGGCTTCGAAGCCGCCGCCTGATACTGGCATTTTGT.AGATGTGGTTT 9951

   39 GACTATTTCTGTATGTCTCCATCTATTGATGAGGGTCTT 1
      |||||||||||||||||||||||||||||||||||||||
 9952 GACTATTTCTGTATGTCTCCATCTATTGATGAGGGTCTT 9990
```

FIGURE 2. Partial nucleotide sequences of two clones isolated by differential screening of the L428 cDNA library compared with sequences in the GeneBank/EMBL data base. Panel A. Sequences of the clone designated H4 (base pairs 3 to 178) shares the greatest level of homology with HLA-Cw2 heavy chain gene sequences (base pairs 4013 to 3840). Panel B. Sequences of the clone designated F2T3 (base pairs 1 to 88) shares the greatest level of homology with the mitochondrial encoded cytochrome oxidase III gene (base pairs 9990 to 9903).

analysis of the autoradiograph with the actual sequence data from the F2T3 clone indicates that these mismatches are potentially due to sequencing errors (data not shown). The numerical positions of the COIII clone are shown in descending order and the sequences shown in this figure are complementary to published sequences of the COIII gene (Montoya *et al.*, 1981; Ojala *et al.*, 1981). These results taken together indicate that the computer generated comparison has aligned the partial sequence of the F2T3 clone with the 3' terminus of the COIII sequence and therefore F2T3 is not likely to be a novel clone.

However, sequence analysis of a third clone, designated ED1T7, suggested that it might be novel. As shown in Figure 3, the partial sequence of this clone between base positions 1 and 104 was most closely related to the *Dictyostelium discoideum* encoded cyclic nucleotide phosphodiesterase between base positions 3486 and 3574. The computer generated comparison shows that only 43 of 104 base pairs of the ED1T7 clone sequence matched the sequence of the phosphodiesterase gene. Because this clone showed less than 50% sequence identity with the D. *discoideum* phosphodiesterase gene, the ED1T7 clone appeared to be novel. A rigorous sequencing analysis was done to confirm this observation. The entire nucleotide sequence was determined by analyzing multiple independently isolated clones from the L428 Hodgkin's-

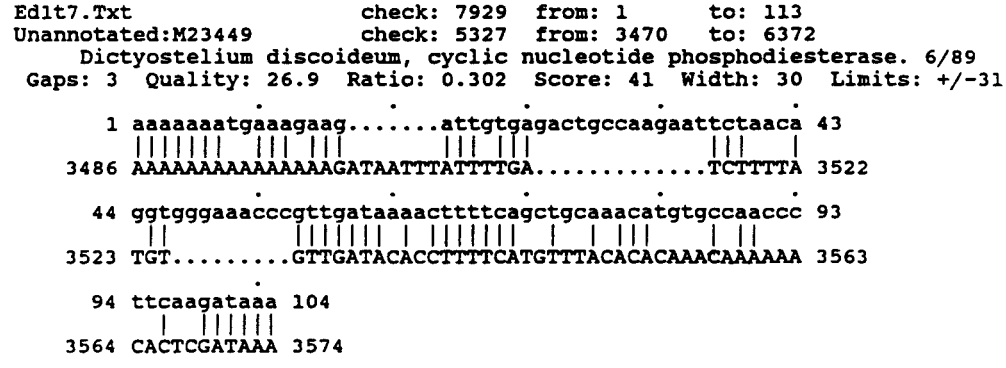

FIGURE 3. Partial sequences of the clone designated ED1T7 (1 to 104) shares the greatest level of homology with the *Dictyostelium discoideum* encoded cyclic nucleotide phosphodiesterase.

```
          10          20          30          40          50          60
           *           *           *           *           *           *
AGT CCC ATC AAG CAG AGA AGT AGA CAG CTT CAG GGG AGG CAG GGG TGA GCA GAG CTG CCA
                                                                      Leu Pro>

          70          80          90          100         110         120
           *           *           *           *           *           *
AGA GCA AAA AGA AAC CAT TCA TAT CAC CTT AGA TAC CAC GGC TCT TCC TAC TCC AGG TGC
Arg Ala Lys Arg Asn His Ser Tyr His Leu Arg Tyr His Gly Ser Ser Tyr Ser Arg Cys>

          130         140         150         160         170         180
           *           *           *           *           *           *
TTC CTG GAG AGG TAT AGA TGC AAA ACT ATA GGA GTC TTT AGA AGA AGC AAC CAG CCG GAC
Phe Leu Glu Arg Tyr Arg Cys Lys Thr Ile Gly Val Phe Arg Arg Ser Asn Gln Pro Asp>

          190         200         210         220         230         240
           *           *           *           *           *           *
TGT CTT GAA ACG CGA TCA GAA AAA GCC AAG AAT AGA GAT GGG GTT GTT CAG GAA AAA TCT
Cys Leu Glu Thr Arg Ser Glu Lys Ala Lys Asn Arg Asp Gly Val Val Gln Glu Lys Ser>

          250         260         270         280         290         300
           *           *           *           *           *           *
GTG AGG ACC CTC TTT TCT GAA TGT GTG AAT CAA TGT GAC ATA CGT AGA AGA CCC ACA AGA
Val Arg Thr Leu Phe Ser Glu Cys Val Asn Gln Cys Asp Ile Arg Arg Arg Pro Thr Arg>

          310         320         330         340         350         360
           *           *           *           *           *           *
TTT TTG AGA ATG TTC TAC CAT CAG AAA CAC TTC CAA CTG GGC CTA AAA GGG ACA GAG ACA
Phe Leu Arg Met Phe Tyr His Gln Lys His Phe Gln Leu Gly Leu Lys Gly Thr Glu Thr>

          370         380         390         400         410         420
           *           *           *           *           *           *
GAA AAA AAT GAA AGA AGA TTG TGA GAC TGC CAG AAT TCT ACA GGT AGG AAA CCC GTT GAT
Glu Lys Asn Glu Arg Arg Leu>

AAA ACT TCT CAG CTG CAA ACA TGT GCC ACC CTT CAA GAT AAA GGA AGC ACC ATC CCG AGA  480
GCA GAG CTG CAG GTA CAG AGG CAG AGG CCC AAG TGT GGG AAC AGA GGA AGA AGT GCA ACC  540
CAT AGG CAC AGA GGC AGA GGC CCA ATC CAT AGT CCA AGA AAG AAG AGC TAC AGG CCC AAA  600
GGG TGG AGC CCT GAG AAA GAG GAT TAC TGC CAG GCC TTG AAA TAT AAC AGA AAT TTG CCT  660
GGC TAG ATT TTG AAA ATG ATT GGG ACA GGA GAC TCC ATT CCA ATT TCC ATT CTC TCC TTT  720
TTG AGA CAG AAA TGT CTG TAA CTG TTA TCC TAT GCC TGT TCC CAC CAT TGT AAT TTG AAA  780
GCA GAT TAA CTT TTC TGT TTC ACT GGT TCT CAG AGG AAG AGG AAT TTG GCC TCA GGA AAG  840
ACC ATA CCC AGA GTT TCA CTC ATA CCT GAT TTA GAT GAT CTA AAT GAT AAG CTT TGG AAC  900
TTT TGA ACC GAT GAT ATT TGA ATG AAA TTT TGG ACT TAG GAT TGA TAT AAT GGG TTG AGA  960
CTT TTG GTA ATG TTA GGA TGG GAT GAA TGT CTT TTA CAC ATG AGA CAG ACA TAA ATT TTG 1020
GGC CAA AGG GCA GAC TCT GGT GGG CTA AGA GTG GCT TCC TAA AAG ACA TAG CCA CAT CCT 1080
AAT CCC CAA AAC CAT GAA TTT ATC TTA TTT GGA GAA GGG GGC CTT GCA GAT GTA ATT AAG 1140
TTA AGA CTC ATG AGG TGA GGA GAT TAT CCT GGA GTA ATC TGG GTA GTC CCT AAA AGT CAT 1200
CAT AAG GAT CCT TAT TAA ACA GAG GCA GAG GCA GAT TAG GTA TAC AGA GGA GGG TGA TGT 1260
GAA GAC ACA GCA GAA AGA GAA ATG TGG CCA TAA GCC AAA AAA TGC CAA TAG CCA CCA AAA 1320
GGT GGA CGT GGC AAG GAA AGA TTC TCC TCT AGA GCC TCT GGA GGG AGC ATG GCT TTG CTG 1380
CCC TTC AAA AAT GTA AGC AAA TAA GTT TCT GTT GTG TTA AGC CAT CAT ATT ATT GGT GGT 1440
AAT TTG TTA CAG CAG CCA AAG GAG ATG ATG GAT GCA ATT AGT TGT TAA TAT TTT AAG GAT 1500
TCT GTA CCT TTT GAA AAT ATT CTG AAG AAA ATC TGC ATA AAT GGT GCT TCT CAT GTG TGG 1560
CAG TAA TAT GGC CAA CAA AAG ACC TTA GTG TAA TTT GGT TTC ATA CTA AAA ATG TCA CTA 1620
TAT ACT TTT TTT GGT GAT ATA CTT TCC AAT CAT CTA AAA CAA AAC TGC AAA TGG CTA GAG 1680
CAG GAC CAT TAC AAA TCT GGG AAA CAA ACC TTT TAA CTC ACT CAA CTA TTG GAA CTT CAT 1740
GAA AGA CCC TGA AAG GTT TTT TTT TTT TAA TGT TTG TGA AAA GCA TGT GGG ATT GCT CTA 1800
AAT AAA AAT ATA TAA GTT AAA AAA                                              1824
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FIGURE 4. Complete nucleotide and predicted amino acid sequence of the ED1 cDNA isolated by differential screening of the Hodgkin's-disease cell line L428 cDNA library. Base pairs 1 through 55 (underlined) were identified using 5' RACE.

disease cell line. The full length clone, designated ED1, which corresponds to base pair positions 56 through 1824 on Figure 4, was compared with the data base. The search again showed no direct match with any sequences which strongly suggested that the cDNA represented a novel gene. The complete nucleotide and predicted amino acid sequence of this cDNA clone is shown in Figure 4. The sequence of the base pairs 1 through 55 were determined using 5' RACE as described below.

C. Characterization of the 5' terminus of ED1

In preliminary sequence analysis I could not identify an ATG translational start site. Therefore, I speculated that the cDNA clone isolated in the differential screening procedure was not full length, lacking the sequences at the 5' terminus containing an ATG start site. Three methods were used to identify and characterize the 5' terminus of the ED1 cDNA. First, the L428 cDNA library was rescreened using ED1 specific probes in order to find longer cDNA clones. In the second approach I attempted to specifically isolate the 5' terminus of the ED1 clone as well as the related clones by RACE (Rapid Amplification of cDNA Ends). Finally, primer extension was done to confirm the position of the 5' terminus.

1. Rescreening the L428 cDNA library

The Hodgkin's disease cDNA library was rescreened using an ED1 specific probe in an effort to identify the most full length ED1 clones. All of the ED1 cDNA clones contained similar 5' termini but still no ATG translational start site was identified. Interestingly, several unique clones with sequences related to ED1 were isolated from the L428 cDNA library. A description of these clones and their potential involvement in the transformed phenotype will be discussed in section IV of the Results.

2. 5' RACE

RACE (Rapid Amplification of cDNA Ends) was used as a second approach to directly isolate the 5' terminus of the ED1 cDNA. Figure 5 illustrates the 5' RACE procedure. In the first step, cDNA synthesis was initiated with a primer (reverse/complementary to base pair positions 657 through 678 of ED1 as shown in Figure 4) specific for the ED1 clone. The design of the this primer was based on sequences obtained from the cDNA clone isolated from the L428 Lambda ZAP library. A poly(dC) tail was added to the 3' terminus of the cDNA molecules. The tailed cDNA molecule was then PCR amplified using a poly(dG) primer and nested primer (reverse/complementary to base pair positions 315 through 334 of the ED1 sequence as shown in Figure 4; this primer has a modified tail that allows for

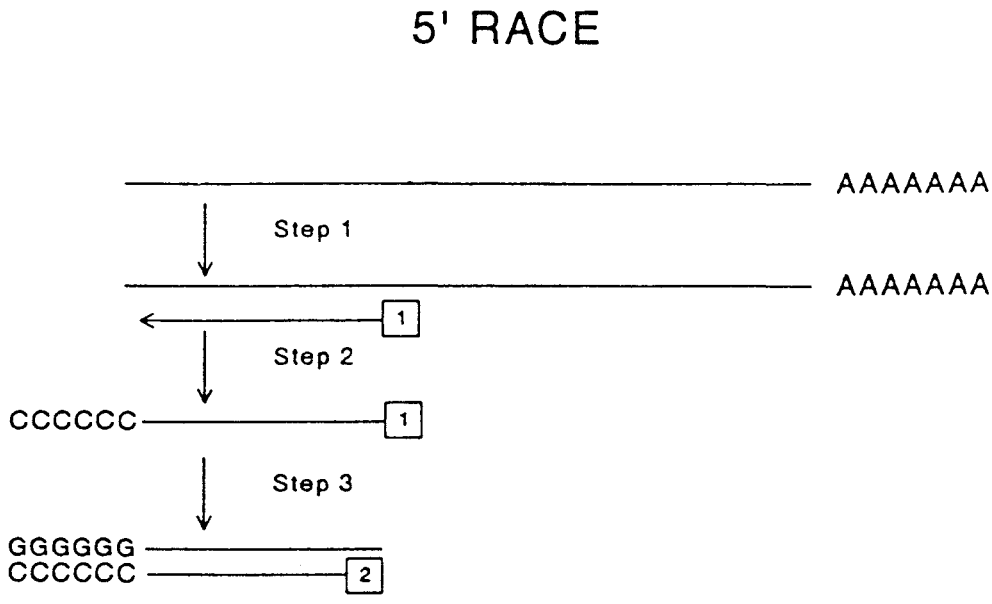

FIGURE 5. Schematic diagram of the 5' RACE procedure used to isolate the 5' terminus of the ED1 cDNA. Rapid Amplification of cDNA Ends (RACE) was used to isolate the 5' terminus of the ED1 cDNA. In this procedure, cDNA synthesis was specifically initiated using an oligomer (primer 1) complementary to the coding strand of the clone. A poly(dC) tail was added to the 3' terminus of the synthesized cDNA clones. The tailed clones are PCR amplified using a primer containing a poly(dG) segment and primer 2 which was complementary to the coding strand. PCR products are subcloned and sequenced as described in Materials and Methods.

rapid cloning into the pAMP-1™ vector which is described in the Materials and Methods section) specific for ED1 sequences. The RACE products were ligated into the pAMP-1 plasmid vector and sequenced. Sequence analysis of the longest RACE clone allowed for the characterization of an additional 55 base pairs of ED1 cDNA sequence (underlined in Figure 4). However, computer analysis showed that this additional sequence did not extend the predicted open reading frame.

3. Primer extension demonstrates heterogeneity of the transcriptional start site

Primer extension analysis was done to help confirm that the sequences identified in the RACE clone represented the 5' terminus of ED1. A primer that initiates reverse transcription from position 126 of the ED1 cDNA (Figure 4) was end labeled. The first strand cDNA products were resolved on a sequencing gel next to a ladder of the ED1 genomic DNA (isolation and characterization of the ED1 genomic clone is discussed in section V of the Results) which show the predicted sequence of the transcript in this region (Figure 6). A primer extension product (indicated by the arrow in Figure 6) migrated at the position of the transcription initiation site as predicted by 5' RACE (Figure 4). An additional five primer extension products were detected.

This figure is representative of results obtained in two replicate experiments.

Figure 7 summarizes the results of the 5' RACE and primer extension experiments. The triangles point to positions on the genomic DNA sequence that are termini of the six primer extension products identified in Figure 6. The arrows indicate the 5' terminal position of 13 clones isolated by 5' RACE as described above. The figure shows that two primer extension products (positions +1 and +8) align with two distinct 5' termini of the ED1 transcripts as predicted by RACE. These data strongly suggest a heterogeneity of transcriptional initiation. The ED1 promoter was determined to be TATA-less (described in section V of the Results). Heterogeneity of transcriptional initiation is a characteristic that has been identified with other genes which are controlled by TATA-less promoters (Kozmik *et al.*, 1992).

FIGURE 6. Primer extension analysis of the ED1 transcriptional start site. cDNA was synthesized from 2 μg of Poly A^+ RNA, isolated from L428 cells using an end labeled primer (the primer sequence is described in Materials and Methods) that initiates reverse transcription at position +120. The synthesized products (lane P_x) were resolved on an 8% urea containing polyacrylamide gel together with the products of a sequencing reaction of genomic DNA representing the corresponding nucleotide sequences. The arrow indicates the position of the predicted transcriptional start site (position +1 as shown on Figure 4) as determined by 5' RACE.

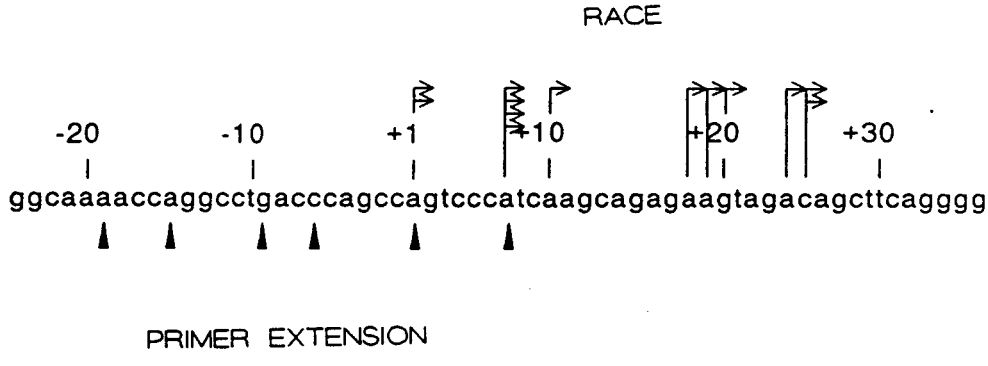

FIGURE 7. Comparison between 5' RACE and primer extension analysis. Arrows show the positions of the 5' terminal nucleotides of 13 clones isolated by 5' RACE. Triangles show the positions of six primer extension products. ED1 sequence containing the transcription initiation regions was obtained from a *Pst* I fragment of a genomic clone which is described in section V of the Results (see figure 22).

II. ANALYSIS OF ED1 TRANSCRIPTION BY RT/PCR

A. Analysis of ED1 expression in association with the activation of normal peripheral blood mononuclear cells

The screening protocol used to isolate ED1 was designed to identify genes that may be involved in the oncogenesis of Hodgkin's disease. Most tumor associated genes (or proto-oncogenes) tend to be involved in the regulation of normal cell growth (Bishop, 1987). Therefore, I evaluated the novel ED1 clone to determine if it fit the second criteria, that the gene be expressed during proliferation, not a housekeeping gene. To do so, I studied ED1 expression in resting and activated peripheral blood mononuclear cells (PBMC). Reverse transcription/polymerase chain reaction (RT/PCR) was used for this study.

The primers used in the PCR reaction amplify a 326-bp segment of ED1 sequence (between base pair positions 4 and 330 as shown in Figure 4) containing most of the open reading frame. In a separated PCR reaction, a 277-bp segment of the beta-2-microglobulin is amplified from cDNA as a control to standardize the amount of cDNA added to the PCR reactions. Additionally, mock reverse transcription reactions containing no AMV-RT were done for each RNA sample tested and amplified to demonstrate that any detectable product was amplified from a cDNA template.

In most assays, the PCR reaction mixture was supplemented with [α^{32}P]-dATP so that the products separated on agarose gels could be identified by autoradiography.

The time course of ED1 expression in activated lymphoid cells was determined by examining cells stimulated in culture with phytohemagglutinin (PHA), a polyclonal T cell mitogen, and collected at various time points following stimulation. The results are shown in Figure 8. The expected 326-bp product was dectected in L428 cells (lane 2). No ED1 was detected in resting PBMCs (lane 4). Upon activation its expression was detectable at seven hours following PHA activation (lane 6), but was not detectable by 24 hours following stimulation (lane 8), nor was it detected in samples harvested at 48 hours (lane 10) or 72 hours (lane 12) after stimulation. Amplification of the 277-bp segment of the Beta-2-microglobulin gene was of similar intensity for all cDNA samples to verify that cDNA was synthesized in every reverse transcription reaction. No specific products were amplified from mock cDNA reactions (odd numbered lanes) verifying that the PCR products detected were specifically amplified from cDNA synthesized in the reverse transcription reaction. This figure is representative of results obtained in seven replicate experiments.

To determine if the cells were activated by stimulation with PHA, [^{3}H]-thymidine incorporation was

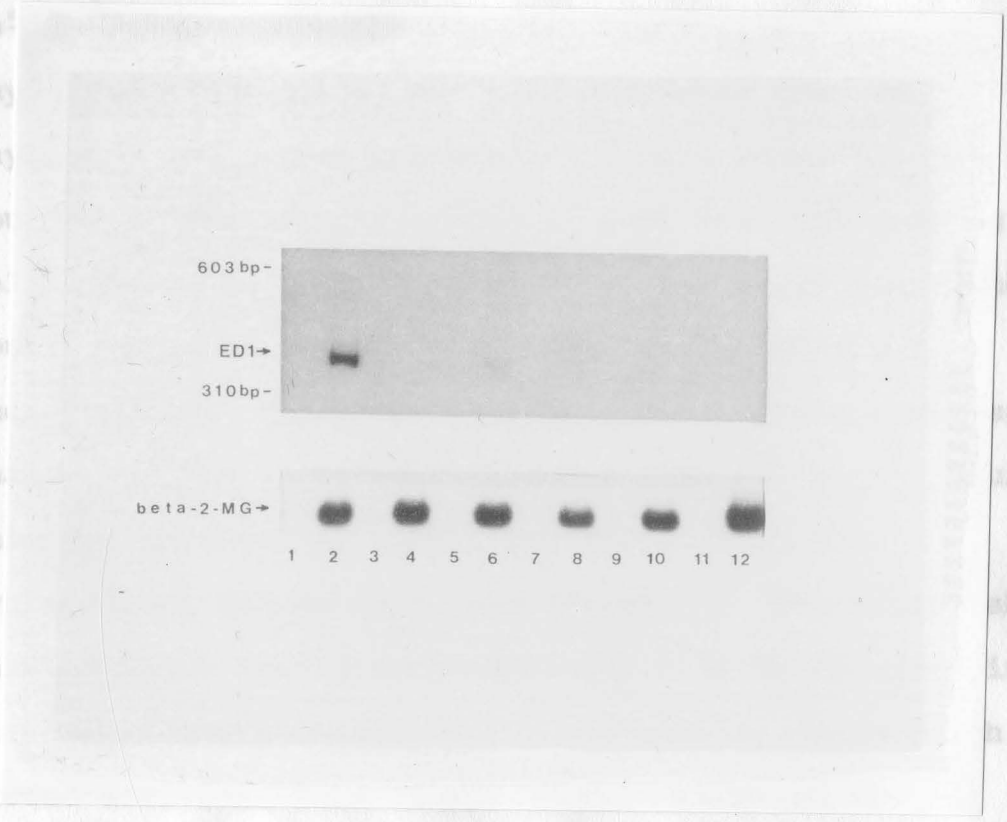

FIGURE 8. Expression of ED1 in normal peripheral blood mononuclear cells (PBMCs) activated with PHA (phytohemagglutinin). The autoradiograph shows results using reverse transcription/polymerase chain reaction (RT/PCR) to measure ED1 and Beta-2-microglobulin transcripts in mRNA isolated from L428 cells (lanes 1 and 2), resting peripheral blood mononuclear cells (lanes 3 and 4), and PBMCs activated with in culture supplemented with PHA (10 ng/ml) for seven hours (lanes 5 and 6), 24 hours (lanes 7 and 8), 48 hours (lanes 9 and 10) and 72 hours (lanes 11 and 12). The cDNA reactions were done with (even lanes) and without (control odd lanes) reverse transcriptase. All reactions were supplemented with 1 μCi [α^{32}P]-dATP (3000 Ci/mmol) and the products were resolved by electrophoresis in a 2% agarose gel. Dried gels were exposed to X-ray film at -85^{o}C.

analyzed to measure DNA synthesis. Stimulated PBMCs collected at the various time points were seeded at 4 x 10^5 cells/ml and pulsed for four hours with [^{3}H]-thymidine. The cells were then harvested and [^{3}H]-thymidine uptake was measured by liquid scintillation counting. The results (Table 2) show that the resting cells and cells stimulated with PHA for seven hours and 24 hours demonstrate background levels of thymidine incorporation. However, [^{3}H]-thymidine uptake increases nearly 40-fold in cells stimulated with PHA for 48 hours. This was an expected result for cells which are synthesizing DNA prior to proliferation. The values shown in this table are representative of results obtained in five replicate experiments. Additionally, cells which become activated form clumps in culture which are observable by light microscopy. Clumping was observed in cells cultured with PHA for at least 24 hours (data not shown). This clumping was not observed in PBMCs cultured for 24 hours in the absence of stimulus. These results strongly suggest that the cells had become activated in response to the PHA stimulation.

The monoclonal antibody anti-human Leu-4 (anti-CD3) was next used to specifically activate T lymphocytes. PBMCs cultured with anti-CD3 were harvested at various time points and RT/PCR was done as described previously. The results are shown in Figure 9. No ED1 expression was

TABLE 2

[^{3}H]-THYMIDINE INCORPORATION

Hours	Mean + SD*
0	80 ± 25
7	211 ± 143
24	174 ± 55
48	7841 ± 893
72	4341 ± 1012

*n = 12 for all time points analyzed

[^{3}H]-Thymidine incorporation values for resting PBMCs and for PBMCs activated with phytohemagglutinin (PHA). Cultures harvested at the indicated time points were pulsed for four hours with 1 μCi [^{3}H]-thymidine per 1 x 10^5 cells. Levels of [^{3}H]-thymidine were determined by liquid scintillation counting. Twelve samples were analyzed for each time point.

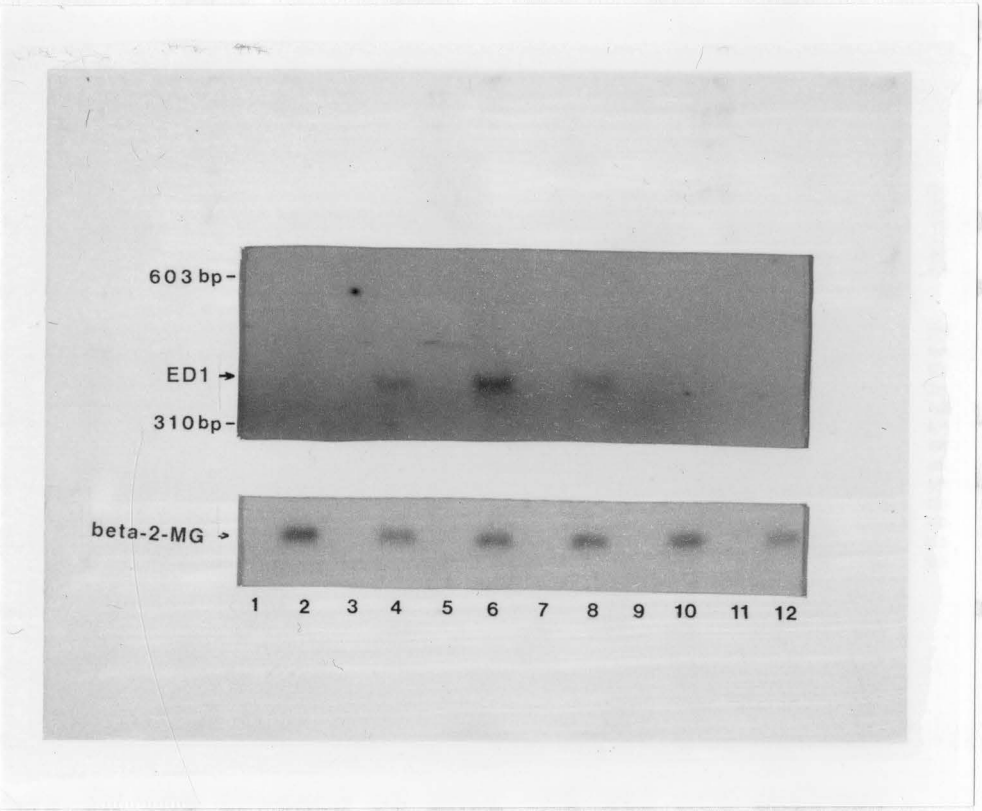

FIGURE 9. Expression of ED1 in normal peripheral blood mononuclear cells activated with anti-CD3 monoclonal antibody. The autoradiograph shows results using RT/PCR to measure ED1 and Beta-2-microglobulin transcripts in mRNA isolated from resting peripheral blood mononuclear cells (lanes 1 and 2), and cells cultured with anti-CD3 for two hours (lanes 3 and 4), four hours (lanes 5 and 6), six hours (lanes 7 and 8), 12 hours (lanes 9 and 10) and 24 hours (lanes 11 and 12). The cDNA reactions were done with (even lanes) and without (control odd lanes) reverse transcriptase. All reactions were supplemented with 1 μCi [α^{32}P]-dATP (3000 Ci/mmol) and the products were resolved by electrophoresis on a 2% agarose gel. Dried gels were exposed to X-ray film at -85^{o}C.

detected in resting cells (lane 2). However, ED1 was expressed in cells stimulated with anti-CD3 for two hours (lane 4), four hours (lane 6) and for six hours (lane 8) but was no longer detectable by 12 hours (lane 10) nor at 24 hours (lane 12) post activation. Amplification of the 277-bp segment of the Beta-2-microglobulin gene was of similar intensity for all cDNA samples. No specific products were amplified from mock cDNA reactions (odd numbered lanes) at detectable levels. This figure is representative of results obtained in four replicate experiments. Cell proliferation was monitored in cells 48 hours after stimulation by [^{3}H]-thymidine incorporation (data not shown). A control was done in which unstimulated cells were cultured for four hours in the absence of anti-CD3. The cells were collected, RNA isolated, and analyzed by RT/PCR. PBMCs culture for four hours in the absence of anti-CD3 did not express detectable levels of ED1 (data not shown).

To more definitively determine the time that ED1 expression initiates, the experiment was repeated with the cells harvested within two hours following activation with anti-CD3. The data in Figure 10 shows that ED1 was not detectable in resting cells (lane 1), or cells harvested at either 15 minutes (lane 2) or 30 minutes (lane 3) following activation with anti-CD3. However, ED1 was detected in cells one hour following stimulation (lane 4)

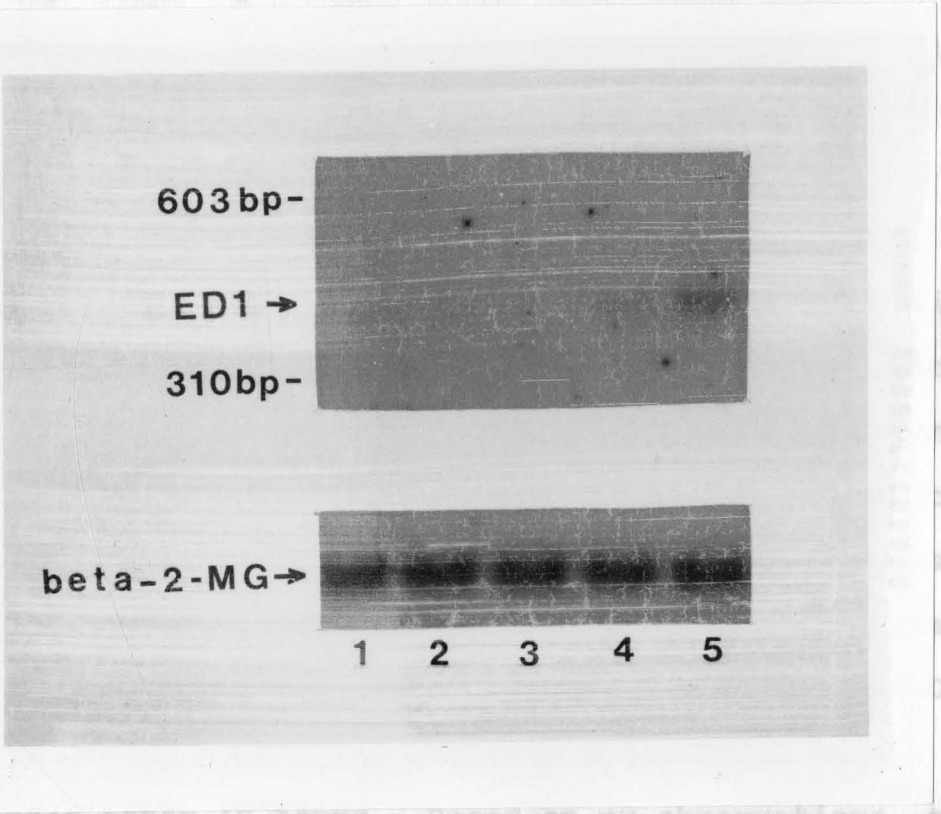

FIGURE 10. Expression of ED1 in normal peripheral blood mononuclear cells activated with anti-CD3. RT/PCR results of ED1 and Beta-2-microglobulin expression in resting cells (lane 1), and cells incubated in culture with anti-CD3 for 15 minutes (lane 2), 30 minutes (lane 3) 60 minutes (lane 4) and 120 minutes (lane 5). All reactions were supplemented with 1 μCi [α^{32}P]-dATP (3000 Ci/mmol) and the products were resolved by electrophoresis on a 2% agarose gel. Dried gels were exposed to X-ray film at -85°C.

and at two hours following stimulation (lane 5). Amplification of the 277-bp segment of the Beta-2-microglobulin gene was of similar intensity for all cDNA samples. No specific products were amplified from mock cDNA reactions at detectable levels (data not shown). This figure is representative of results obtained in two replicate experiments.

A recent classification scheme devised by Gerald Crabtree has categorized genes which are induced to expression during T cell activation (Crabtree, 1989; Altman *et al.*, 1990). Over 100 genes have been identified that are considered to be T cell activation genes. These genes are grouped into three major categories based on when their expression is initiated following activation; the immediate early activation genes, which are expressed within the first 30 minutes following stimulation; the early activation genes, which are expressed between 30 minutes and 48 hours; and the late genes which are expressed after 48 hours. Based on my observations, the novel clone should be categorized as an early activation gene and therefore, we named it ELAG (Early Lymphoid Activation Gene).

B. ELAG Expression in activated Jurkat cells

To help confirm that ELAG (Early Lymphoid Activation Gene) is expressed in activated T cells, studies were done

to evaluate ELAG expression in the Jurkat leukemic T cell line. Jurkat cells are an accepted tissue culture line for studying T cell activation because upon stimulation with two signals, PHA (phytohemagglutinin) and TPA (12-*O*-tetradecanoyl phorbol-13-acetate), these cells mimic several responses observed in normal activated T cells (Gillis and Watson, 1980). Specifically, activated Jurkat cells produce and secrete IL-2 in an analogous manner to normal activated T cells. Therefore, these studies would help to confirm the involvement of T cells and provide information about the signals required for ELAG expression. The results in Figure 11 show that ELAG expression was not detected in Jurkat cells unstimulated in culture for four hours (lane 1) or 24 hours (lane 5). ELAG expression was detected in cells cultured for four hours with either PMA (lane 2) or PHA (lane 3). Jurkat cells stimulated with both PHA and PMA for four hours expressed ELAG (lane 4) at greater levels than were detected when cells were stimulated with either PMA or PHA alone. Jurkat cells cultured with PHA and PMA for 24 hours express barely detectable levels of ELAG (lane 6). This figure is representative of results obtained in two replicate experiments. IL-2 synthesis in resting and stimulated cells was directly measured by ELISA to determine if the cells were activated. The results showed that unstimulated cells, or cells stimulated with only PHA

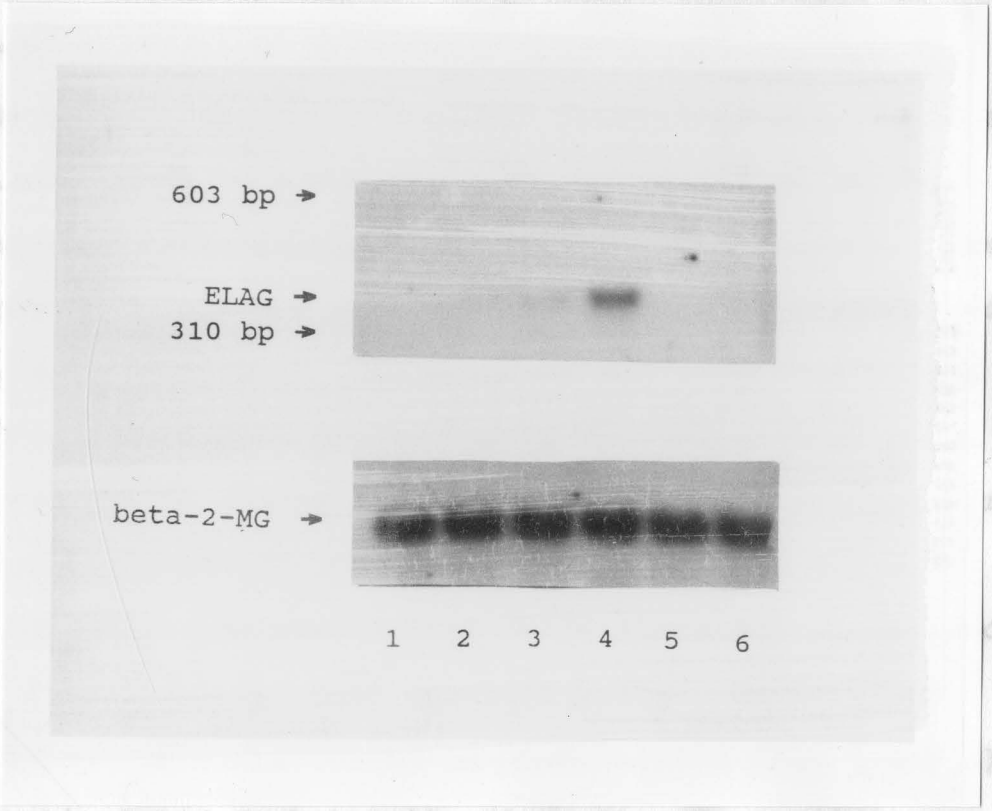

FIGURE 11. RT/PCR analysis of ELAG expression in non-activated and activated Jurkat cells. ELAG expression in non-activated and activated Jurkat cells. RT/PCR was used to measure ELAG and Beta-2-microglobulin expression in the Jurkat T cell line cultured for four hours in medium alone (non-activated, lane 1), or medium supplemented with PHA (lane 2), PMA (lane 3), or PHA and PMA (lane 4), and cells cultured for 24 hours in medium alone (lane 5) or with both PHA and PMA (lane 6). All reactions were supplemented with 1 μCi [α^{32}P]-dATP (3000 Ci/mmol) and the products were resolved by electrophoresis on a 2% agarose gel. Dried gels were exposed to X-ray film at -85^{o}C.

or only PMA did not synthesize IL-2. However, Jurkat cells stimulated with both PHA and PMA secreted IL-2 into the culture supernatants (data not shown).

C. PCR products hybridize with an ELAG specific probe

To determine if the products identified in these RT/PCR experiments represented ELAG sequences, PCR products were tested by Southern hybridization. To do so, cDNA was added to a PCR reaction and amplified in the absence of radio-labeled nucleotides. The products were separated by agarose gel electrophoresis and transferred to a nylon membrane. The membrane was incubated with a [α^{32}P]-labeled ELAG probe, washed and autoradiographed. The results in Figure 12 show that amplified products from L428 cells (lane 2), anti-CD3 activated PBMCs (lane 6), and PHA/PMA stimulated Jurkat cells (lane 10) hybridized with a radiolabeled ELAG specific probe. However, no hybridization was detected in unstimulated PBMC (lane 4) or unstimulated Jurkat cells (lane 8). No specific products which were amplified from mock cDNA reactions hybridized with the ELAG probe at detectable levels (odd numbered lanes). This figure is representative of results obtained in three replicate experiments. These results confirm that ELAG transcripts are expressed upon activation of PBMCs or Jurkat cells.

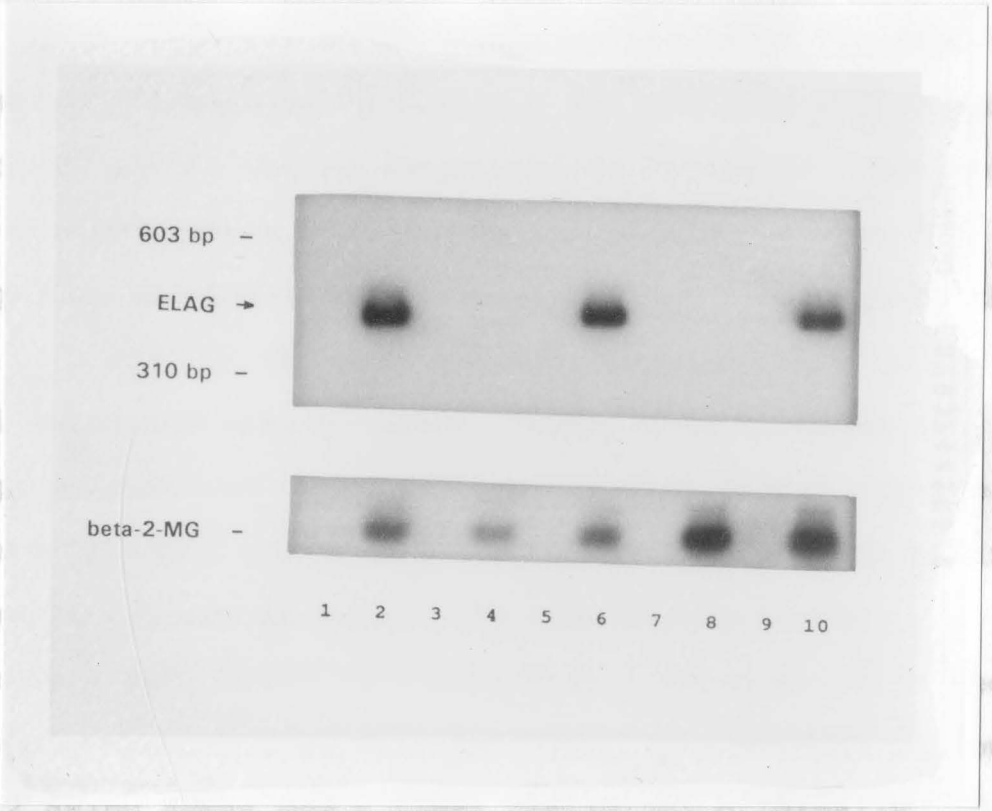

FIGURE 12. Hybridization of PCR amplified products with an ELAG specific probe. RT/PCR products from various cultured cells were separated by electrophoresis through 1% agarose gel, transferred to a nylon membrane and incubated with either a ^{32}P-labeled ELAG probe or a Beta-2-microglobulin probe. The RT/PCR products analyzed were from L428 cells (lanes 1 and 2), resting PBMCs (lanes 3 and 4), PBMC cultured for four hours with anti-CD3 antibody (lanes 5 and 6), non-activated Jurkat cells (lanes 7 and 8) and PHA/PMA activated Jurkat cells (lane 9 and 10). The cDNA reactions were done with (even lanes) and without (control odd lanes) reverse transcriptase. The membrane was washed and autoradiographed at -85^{o}C.

III. Properties of the putative ELAG-encoded proteins

A. Amino acid sequence and putative structure of the ELAG-encoded protein

The predicted amino acid composition of the ELAG open reading frame shown in Figure 4 was analyzed for consensus protein motifs and is diagrammed in Figure 13. Beginning at the N-terminus, the predicted protein contains the sequence Pro-Arg-Ala-Lys-Arg (positions 2 through 6) that is very similar to known nuclear localization signals (Dingwall and Laskey, 1986). Also, this terminus is highly basic and lacks any identifiable signal peptide. The C-terminus contains a single zinc finger-like motif that is related to the TFIIIA zinc finger domain ($Cx_3Cx_{12}Hx_2H$; cysteine followed by 3 amino acids, a second cysteine followed by 12 amino acids, a histidine followed by 2 amino acids and a final histidine) originally described in Xenopus (Miller *et al.*, 1985; Brown *et al.*, 1985). The computer also identified four potential sites of phosphorylation. There are two serine residues (positions 48 and 65) and one threonine residue (position 102) that are potential substrates of protein kinase-C. There is also one serine residue (position 9) that is a potential phosphorylation substrate of c-*raf*. This analysis suggest that the ELAG encodes a nuclear

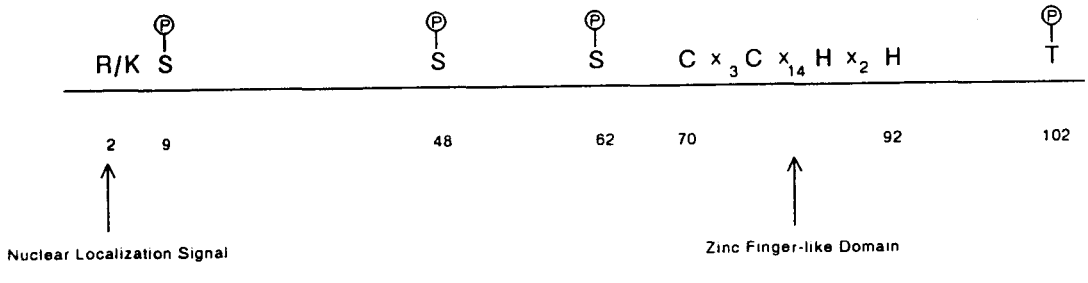

FIGURE 13. Structural motifs encoded by the predicted ELAG-encoded protein. This diagram shows the protein motifs for the deduced open reading frame based on the ELAG cDNA sequence (formerly identified as ED1) shown in Figure 4. R/K putative nuclear localization signal Pro-Arg-Ala-Lys-Arg; S, potential serine sites of phosphorylation; T, potential sites of threonine phosphorylation; $Cx_3Cx_{12}Hx_2H$, TFIIIA-like zinc finger domain.

phosphoprotein utilizing a single zinc finger which potentially binds DNA or RNA.

B. In vitro translation of the ELAG open reading frame

To investigate the possibility that ELAG utilizes a CUG translation initiation codon instead of an AUG, I tested the ability of the ELAG open reading frame to be translated in vitro from sense transcripts encoding wild type ELAG sequences versus sense transcripts containing a mutation at the putative translation start site. To do so, I used a PCR based protocol to create the mutation at the putative translational start site to test the hypothesis that the CUG (CTG in the cDNA sequence) at position 55 (see Figure 4) is necessary for translational initiation. The mutational process is illustrated in Figure 14. Briefly, three PCR reactions are performed using four different primers. One pair of primers (primers B and C) are complementary to each other. These primers are identical to the double stranded DNA positions 43 to 67 except that the CTG at position 55 was changed to TTA on primer C. Likewise, the CAG (reverse complimentary to CTG) was changed to TAA (reverse complimentary to TTA) on primer D. In the first two PCR reactions, primers A and B were used in a single reaction while primers C and D are used in the other reaction. The expected products

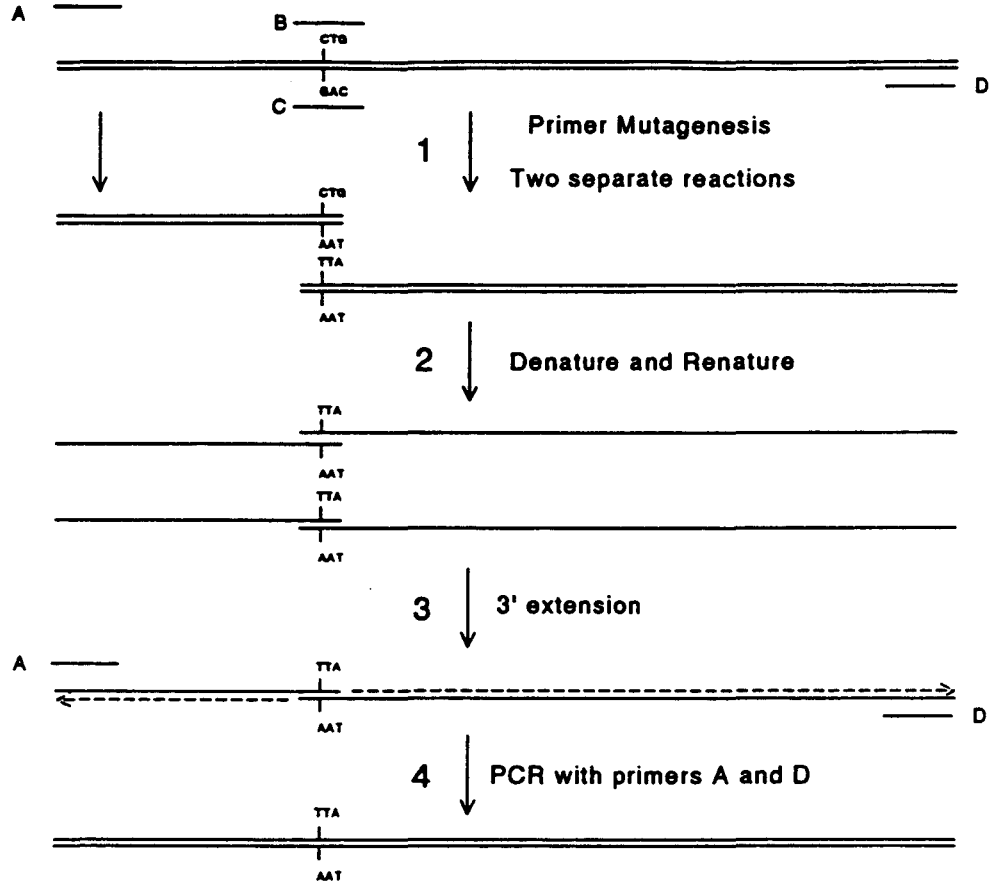

FIGURE 14. Schematic diagram of the procedure used to induce mutations in the putative ELAG translational initiation site. The scheme to mutate the putative CUG translational start codon to a UUA was done by a two step PCR amplification. In the first step, two PCR products are synthesized. One product was amplified between Primer A and primer C. The other was amplified between primer B and primer D. Primers B and C encode a two base mismatch that will be incorporated into the final product. The products are purified to remove excess primers and added to the PCR reaction in the second step in which amplification takes place between primers A and D and would incorporate the double base mismatch from step 1.

would overlap and contain the substitutions that allow for the CTG to TTA change. The products of each of the reactions were purified to remove the primers. The product are combined in a single PCR reaction and amplified using primers A and D. The final product would contain the sequence change in the full length clone. The PCR products were then ligated into the pGEM^{TM} PCR cloning vector both under control of the T7 RNA polymerase promoter. The mutation was designed to alter the CTG at the putative translational start site to a TTA. Both CTG and TTA encode leucine so the change will not affect the peptide sequence. Sequence analysis of the mutant and wild type ELAG clones (Figure 15) shows the mutation at positions 55 and 57 to a T and A respectively.

Sense transcripts from the wild type and mutant cDNA inserts were synthesized in vitro and analyzed by electrophoresis through a formaldehyde containing agarose gel and stained with ethidium bromide to determine the relative concentration of transcripts produced by each transcription reaction. Equal amounts of wild type and mutant transcripts were added to a wheat germ in vitro translation system. The translation products were separated by SDS-PAGE. As a control, an in vitro translation reaction was done in which no RNA was added. The results in Figure 16 show that a 13-kDa protein was translated in the reaction supplemented with ELAG sense

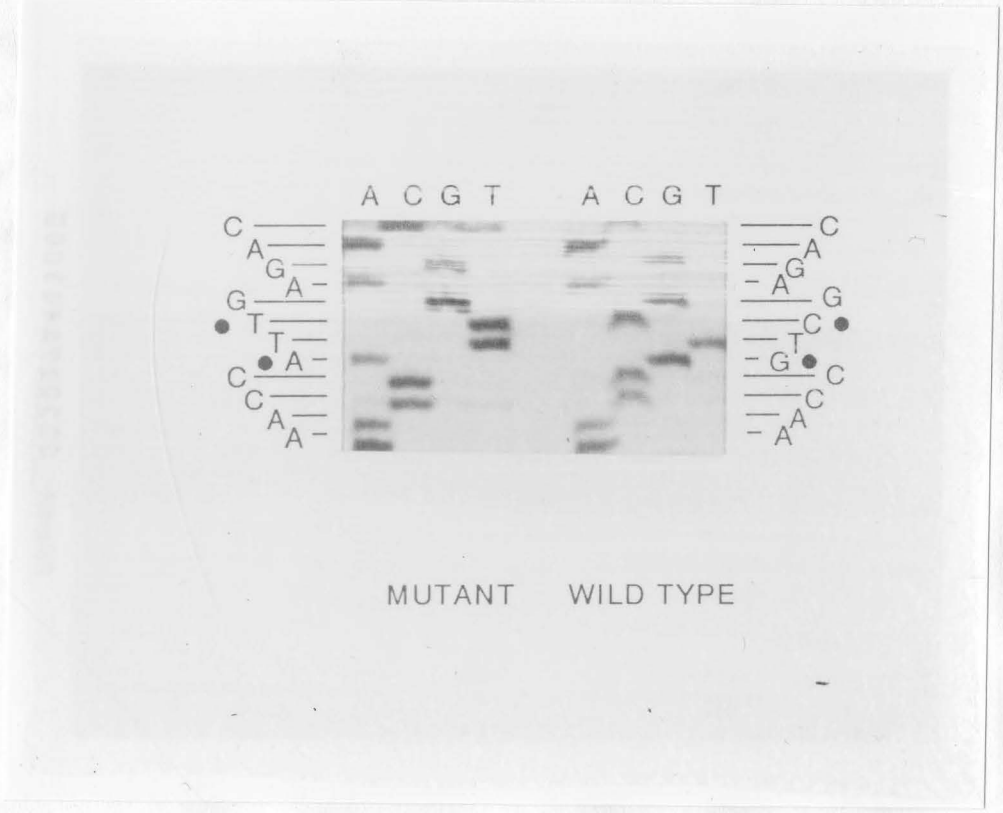

FIGURE 15. The autoradiograph shows the nucleotide sequence of the wild type ELAG cDNA compared to the cDNA with the mutated translational start site. The Sanger sequencing of the subclone mutant PCR fragment is compared to the wild type sequence. The mutations C to T and G to A are noted with the symbol (●).

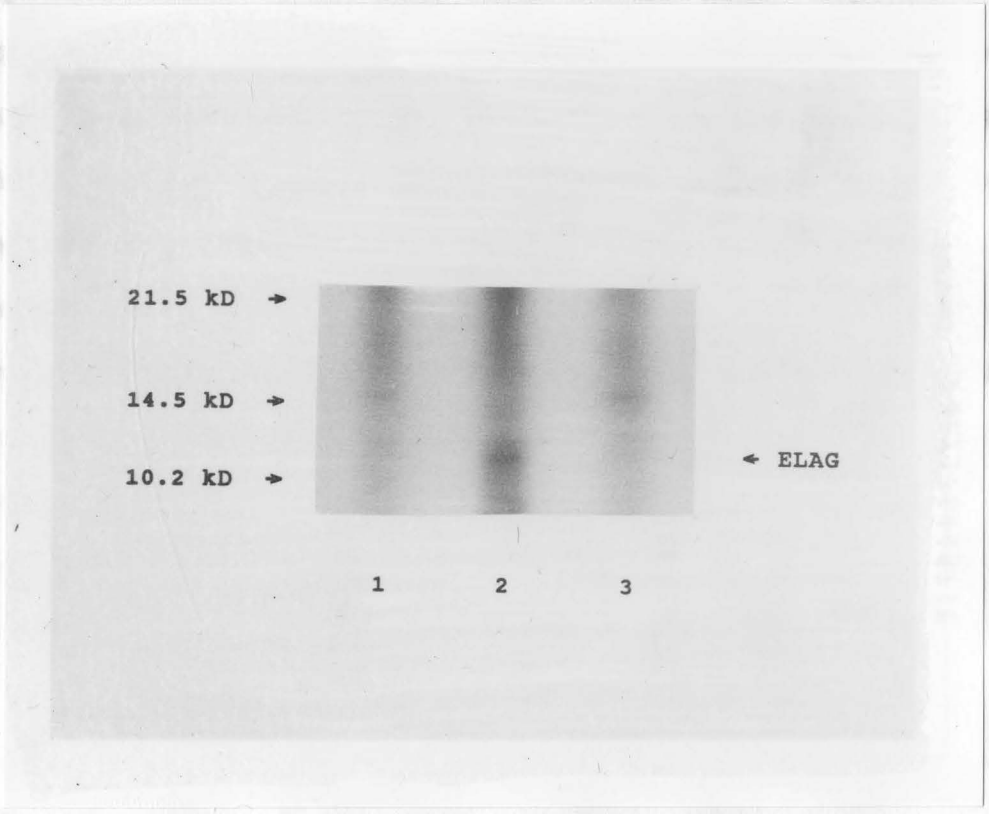

FIGURE 16. In vitro translation of wild type and mutant ELAG transcripts. The figure shows an autoradiograph of an SDS-polyacrylamide gel analyzing wheat germ in vitro translation reaction containing no RNA (lane 1), in vitro synthesized transcripts encoding wild type ELAG 3 (lane 2), and in vitro synthesized transcripts encoding ELAG 3 in which the CUG start codon was mutated to a UAA (lane 3).

RNA (lane 2) that was not present in the mock translation (lane 1). This data is consistent with computer analysis of the ELAG open reading frame which predicts that ELAG encodes a 13.2-kDa protein. The translation reactions that received the mutant transcript (lane 3) shows no detectable levels of a 13.2-kDa protein. This figure is representative of results obtained in three replicate experiments. The data shows that the ELAG-encoded open reading frame, without an in-frame AUG, can be translated. Additionally, by altering the CUG at sequence position 55 reduces the translational efficiency to undetectable levels, thus indicating that the CUG is the utilized translational initiation site for ELAG-enocded protein.

IV. Characterization of the transformation potential of ELAG

A. Rationale

The results described in the previous section demonstrate that ELAG is expressed in association with normal lymphoid cell growth and proliferation. This is a characteristic common to the proto-oncogenes in that they are normally involved in the regulation of cell growth and proliferation (Bishop, 1987). Therefore, ELAG is a candidate gene, like a proto-oncogene, such that when perturbed could be involved in the transformed phenotype. Proto-oncogenes become involved in the development of the transformed phenotype by two major mechanisms: 1) mutation which alters the open reading frame and subsequently changes the structure and activity of the protein, or 2) the proto-oncogene becomes over-expressed (Bishop, 1987). I was interested in determining if either alteration potentially affects ELAG in Hodgkin's-disease cells or other tumor types.

B. Analysis of potential mutations associated with ELAG expressed in the Hodgkin's disease L428 cells

In order to identify any potential tumor specific mutations in ELAG, it was necessary to obtain and sequence cDNA clones from normal activated PBMCs and compare them

with the sequences of the L428 derived clones. Clones were isolated from activated PBMCs using 3' RACE (Rapid Amplification of cDNA Ends) as illustrated in Figure 17. Poly A^+ RNA isolated from anti-CD3 activated PBMCs and used as a template in a cDNA reaction. Synthesis of cDNA was initiated by priming with a modified poly(dT) primer (described in Materials and Methods) that has a specifically engineered tail at the 5' terminus. The cDNA was amplified in a PCR reaction containing a modified ELAG-specific primer (ELAG sequences are identical to positions 64 through 85 shown on Figure 18; this primer has a modified tail that allows for rapid cloning into the pAMP-1^{TM} vector which is described in the Materials and Methods section), and a primer which is reverse complementary to the tail on the modified poly(dT) primer (described in Materials and Methods). The RACE products were subcloned into pAMP-1^{TM} using procedures described in the Materials and Methods. The clones were isolated by hybridization using a [^{32}P]-labeled ELAG specific probe.

Clones isolated by this method were sequenced in both directions and are shown in Figure 18 (positions 64 to 667). Surprisingly, this new clone contained sequences not encoded by the ED1-ELAG clone shown in Figure 4. In order to characterize the entire clone, 5' RACE was done (described previously as shown in Figure 5) using a primer

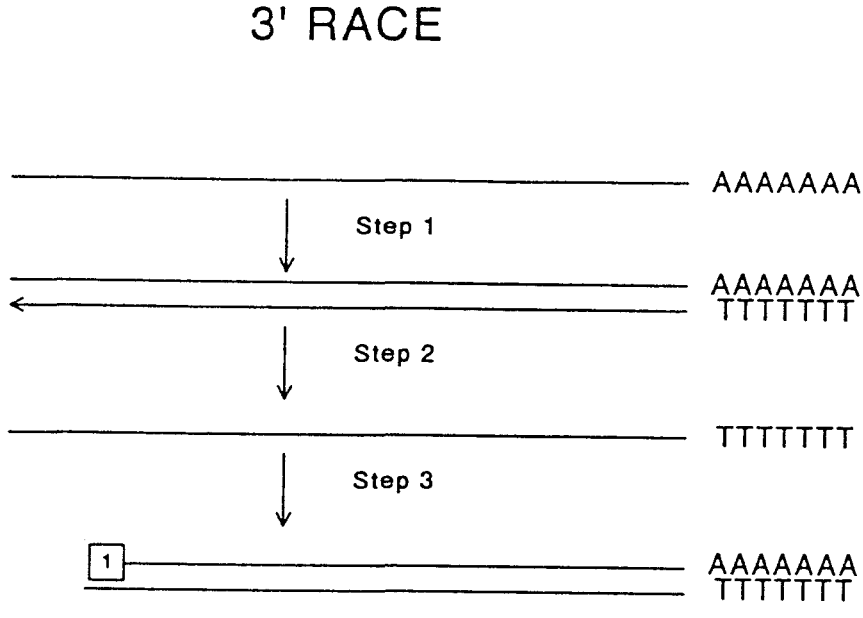

FIGURE 17. Schematic diagram of the 3' RACE procedure used to isolate ELAG clones from normal activated PBMCs and L428 cells. Reverse transcription of RNA isolated from either L428 cells or PBMCs activated in culture for four hours with anti-CD3 monoclonal antibody was initiated with oligo(dT). The cDNA was amplified using a poly(dA) primer and primer 1 which is identical to the coding strand of the ELAG and positioned near the amino terminus of the open reading frame. The PCR products are subcloned and sequenced as described in Materials and Methods.

```
          10          20          30          40          50          60
           *           *           *           *           *           *
AGT CCC ATC AAG CAG AGA AGT AGA CAG CTT CAG GGG AGG CAG GGG TGA GCA GAG CTG CCA    Normal
AGT CCC ATC AAG CAG AGA AGT AGA CAG CTT CAG GGG AGG CAG GGG TGA GCA GAG CTG CCA    Hodgkin's

          70          80          90          100         110         120
           *           *           *           *           *           *
AGA GCA AAA AGA AAC CAT TCA TAT CAC CTT AGA TAC CAC GGC TCT TCC TAC TCC AGG TGC    Normal
AGA GCA AAA AGA AAC CAT TCA TAT CAC CTT AGA TAC CAC GGC TCT TCC TAC TCC AGG TGC    Hodgkin's

          130         140         150         160         170         180
           *           *           *           *           *           *
TTC CTG GAG AGG TAT AGA TGC AAA ACT ATA GGA GTC TTT AGA AGA AGC AAC CAG CCG GAC    Normal
TTC CTG GAG AGG TAT AGA TGC AAA ACT ATA GGA GTC TTT AGA AGA AGC AAC CAG CCG GAC    Hodgkin's

          190         200         210         220         230         240
           *           *           *           *           *           *
TGT CTT GAA ACG CGA TCA GAA AAA GCC AAG AAT AGA GAT GGG GTT GTT CAG GAA AAA TCT    Normal
TGT CTT GAA ACG CGA TCA GAA AAA GCC AAG AAT AGA GAT GGG GTT GTT CAG GAA AAA TCT    Hodgkin's

          250         260         270         280         290         300
           *           *           *           *           *           *
GTG AGG ACC CTC TTT TCT GAA TGT GTG AAT CAA TGT GAC ATA CGT AGA AGA CCC ACA AGA    Normal
GTG AGG ACC CTC TTT TCT GAA TGT GTG AAT CAA TGT GAC ATA CGT AGA AGA CCC ACA AGA    Hodgkin's

          310         320         330         340         350         360
           *           *           *           *           *           *
TTT TTG AGA ATG TTC TAC CAT CAG AAA CAC TTC CAA CTG GGC CTA AAA GGG ACA GAG ACA    Normal
TTT TTG AGA ATG TTC TAC CAT CAG AAA CAC TTC CAA CTG GGC CTA AAA GGG ACA GAG ACA    Hodgkin's

          370         380         390         400         410         420
           *           *           *           *           *           *
GAA AAA AAA TGA AAG AAG ATT GTG AGA CTG CCA GAA TTC TAC AGT GTA TGC AAT GGT ATG    Normal
GAA AAA AAA TGA AAG AAG ATT GTG AGA CTG CCA GAA TTC TAC AGT GTA TGC AAT GGT ATG    Hodgkin's

          430         440         450         460         470         480
           *           *           *           *           *           *
AGG AAC TGT TCC ACA TTA AAG GAG ACT AAG AAG ACA TAA CAA CTC GAT GCA ATG TGT GAT    Normal
AGG AAC TGT TCC ACA TTA AAG GAG ACT AAG AAG ACA TAA CAA CTC GAT GCA ATG TGT GAT    Hodgkin's

          490         500         510         520         530         540
           *           *           *           *           *           *
TCT GGA CTG GAT CTT GCA TTG GAG GAG AAA ATG CTA TAA AAG GTA TCA CTG AGA CAG TTA    Normal
TCT GGA CTG GAT CTT GCA TTG GAG GAG AAA ATG CTA TAA AAG GTA TCA CTG AGA CAG TTA    Hodgkin's

          550         560         570         580         590         600
           *           *           *           *           *           *
ACA TCA TTG GAA CAT GGA TGG TTG ATT AGA TGG AAG TAT TAC ATT AAT GAT ACA TTT TCC    Normal
ACA TCA TTG GAA CAT GGA TGG TTG ATT AGA TGG AAG TAT TAC ATT AAT GAT ACA TTT TCC    Hodgkin's

          610         620         630         640         650         660
           *           *           *           *           *           *
TGA ATT TGG TAA CTT TGC TAT TGT TAC CTA CAC GAA TAC TCT GCT CTA GGA AAC AGT AAT    Normal
TGA ATT TGG TAA CTT TGC TAT TGT TAC CTA CAC GAA TAC TCT GCT CTA GGA AAC AGT AAT    Hodgkin's

    665
     *
GAG ATA A      Normal
GA             Hodgkin's
```

FIGURE 18. Comparison of the complete nucleotide sequences of the ELAG-related cDNA clone isolated from activated PBMCs (Normal) versus the ELAG-related clone isolated from L428 cells (Hodgkin's). 5' RACE and 3' RACE clones were isolated using mRNA from L428 cells or from PBMCs collected four hours after activation with anti-CD3. The sequences underlined indicate the only region of divergence between the two cDNA clones.

(reverse/complementary to sequences at positions 488 through 505 on Figure 18) to initiate reverse transcription. The poly(dG) tailed cDNA products were amplified as using a modified poly(dC) primer and a modified primer specific to the ELAG-related clone (reverse complementary to positions 462 through 481 as shown on Figure 18; this primer has a modified tail that allows for rapid cloning into the pAMP-1^{TM} vector which is described in the Materials and Methods section). The sequences obtained from this analysis showed that the 5' terminus of the ELAG-related clone was identical to the 5' terminus of the ED1-ELAG cDNA shown in Figure 4.

Since my original objective in this study was to search for mutations in the L428 associated clone, it was necessary to repeat the 5' and 3' RACE procedure using RNA isolated from L428 cells in an effort to isolate a clone similar to ELAG-related clone from activated PBMCs. Such a clone was isolated from L428 cells and sequenced. A comparison of the ELAG clone isolated from normal activated PBMCs versus the Hodgkin's-disease L428 tumor cell line is shown in Figure 18. The results show that the sequences of the ELAG-related clone are nearly identical. The only differences identified are at the 3' terminus. The cDNA clone isolated from normal activated PBMCs has an additional five base pairs (underlined in Figure 18) not identified on the tumor associated ELAG

clone. It is possible that these differences are the result of a cloning artifact and do not represent actual differences in the sequences of the transcripts. No sequence divergence was detected in the computer predicted open reading frame. Therefore, the ELAG-related clones isolated from activated PBMCs and L428 cells would encode the same protein.

C. ELAG family members from L428 cells

To date, four distinct but related clones have been isolated by the RACE and rescreening the library. These clones have all been sequenced and comparison is shown in Figure 19. The ED1-ELAG clone originally isolated from the Hodgkin's disease L428 cell line by differential screening (Figure 4) is now designated as ELAG 1. ELAG 1 and ELAG 2 were isolated only from the L428 Lambda ZAP™ library. The ELAG-related clone isolated from activated PBMCs and L428 cells (Figure 18) is now designated as ELAG 3. ELAG 3 was only isolated by RACE. ELAG 4 was isolated from both the cDNA library and by RACE.

Eight regions of sequence identity (regions A through H) have been identified (Figure 19). All segments represented by a given letter share 100% sequence identity. All clones identified to date encode region A. ELAG clones 1, 2, and 3 contain region B. Region C is unique to ELAG 1, while regions D and G are unique to ELAG

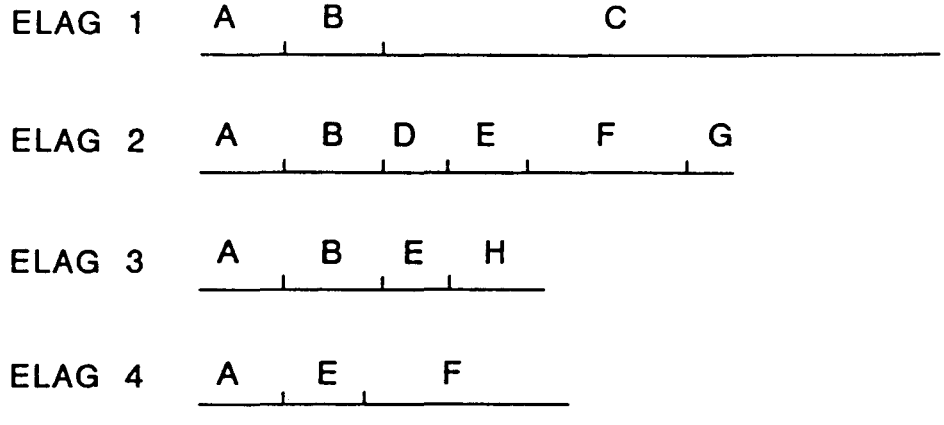

FIGURE 19. Comparison of the sequences of the four ELAG cDNA clones isolated from the L428 cell line. The sequence identity shared by each clone is displayed with a letter. The 13.2-kDa open reading frame is encoded in regions A and B.

2 and region H is unique to ELAG 3. No unique regions were identified in ELAG 4.

The open reading frame identified by computer analysis in ELAG 1 (Figure 4) is encoded in regions A and B. Therefore, ELAG clones 1, 2, and 3 all contain the same open reading frame, but have different 3' termini. ELAG 4 has an open reading frame that is terminated shortly after region A. Interestingly, the open reading frame contained in region B encodes a zinc finger motif, and therefore ELAG 4 protein would not exhibit this domain.

Northern blot analysis was done using restriction fragments of ELAG clones as probes to dispel any possibility that the ELAG members were cloning artifacts. Figure 20A shows a partial restriction enzyme digestion map of the ELAG clones used to prepare probes for this study. Three probes were used: 1) a 340-bp *Eco*R I fragment of the ELAG 1 clone which contains regions A and B and therefore is expected to hybridize to all ELAG transcripts, 2) a 668-bp *Bam*H I fragment of ELAG 1 which contains only region C and therefore should be specific for ELAG 1, and 3) a 1.0-kb *Eco*R I fragment of ELAG 2 which contains regions D, E, F and G and therefore is expected to hybridize to ELAG 2, ELAG 3 and ELAG 4 transcripts.

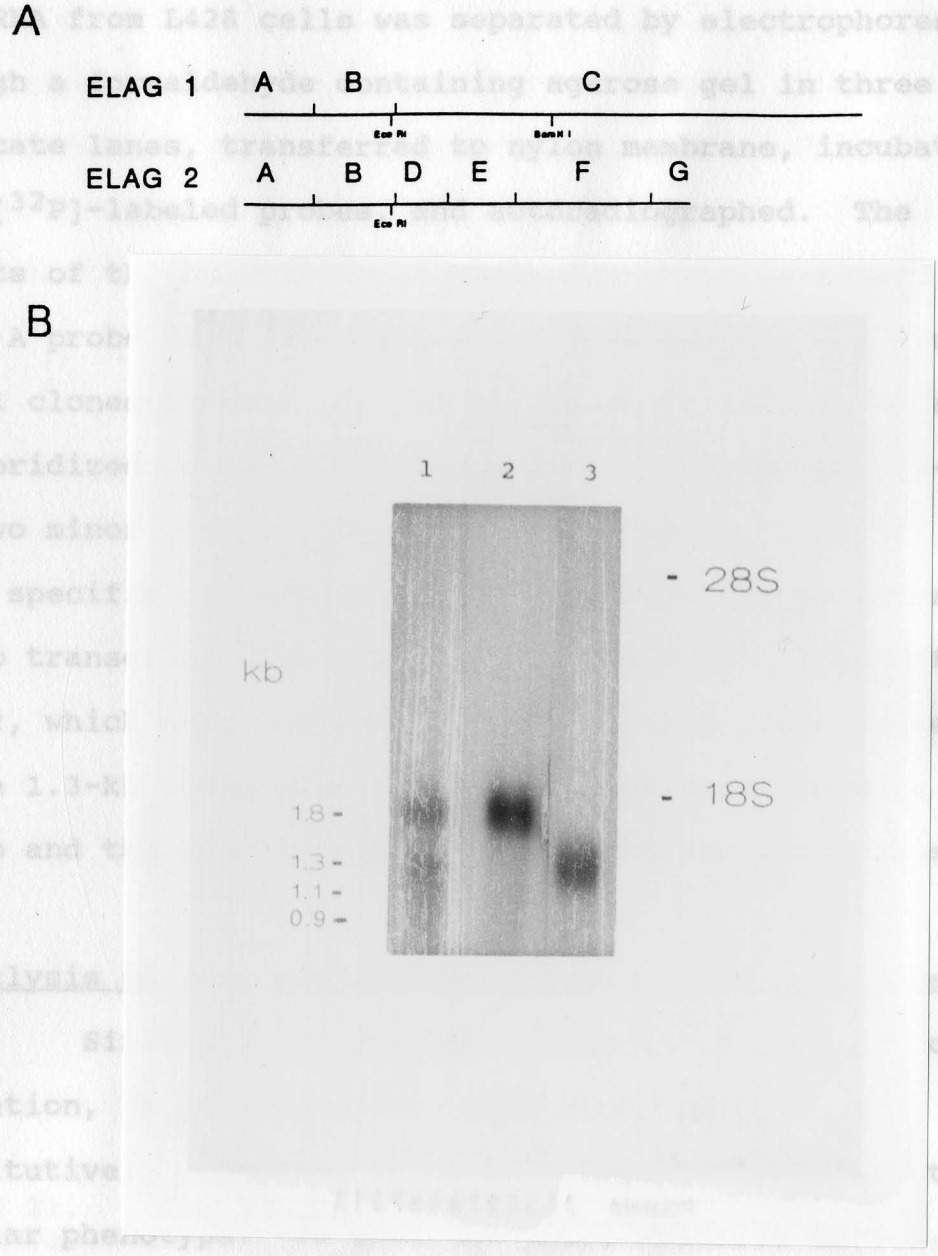

FIGURE 20. Northern blot analysis of RNA isolated from L428 cells using restriction fragments of ELAG cDNA clones as probes. Panel A show partial restriction maps of ELAG 1 and ELAG 2. Panel B shows the autoradiographs of RNA hybridized with the following probes: lane 1, a 340-bp *Eco*R I fragment of ELAG 1 containing regions A (common to all ELAG clones identified to date) and B; lane 2, a 668-bp *Bam*H I fragment of ELAG 1 containing the 3' terminal portion of the region C which is ELAG 1 specific; and lane 3, a 1.1-kb *Eco*R I fragment of ELAG 2 which contains regions D, E, F and G.

RNA from L428 cells was separated by electrophoresis through a formaldehyde containing agarose gel in three replicate lanes, transferred to nylon membrane, incubated with [^{32}P]-labeled probes, and autoradiographed. The results of the hybridization study are shown in Figure 20B. A probe containing sequences from regions A (common to all clones identified) and B (common to ELAG 1, 2, and 3) hybridized to two major transcripts, 1.8-kb and 1.3-kb, and two minor transcripts, 1.1-kb and 0.9-kb (lane 1). A probe specific for ELAG 1, hybridized only to the upper, 1.8-kb transcript (lane 2). The 1.0-kb *Eco*R I fragment of ELAG 2, which contains regions D, E, F, and G hybridized to the 1.3-kb transcript (lane 3). Hybridization with the 1.1-kb and the 0.9-kb transcripts are barely detectable.

D. Analysis of ELAG expression in human tumor cell lines

Since ELAG expression is regulated during T cell activation, it is a candidate gene that when constitutively expressed or over-expressed may affect the cellular phenotype. To test the third criteria that I established for evaluating clones isolated by differential screening, I wanted to determine if ELAG is aberrantly expressed in association with the development of the transformed phenotype. To do so, several tumor cell lines were tested for ELAG expression by RT/PCR. The data in Figure 21 show that the Hodgkin's-disease cell line L428

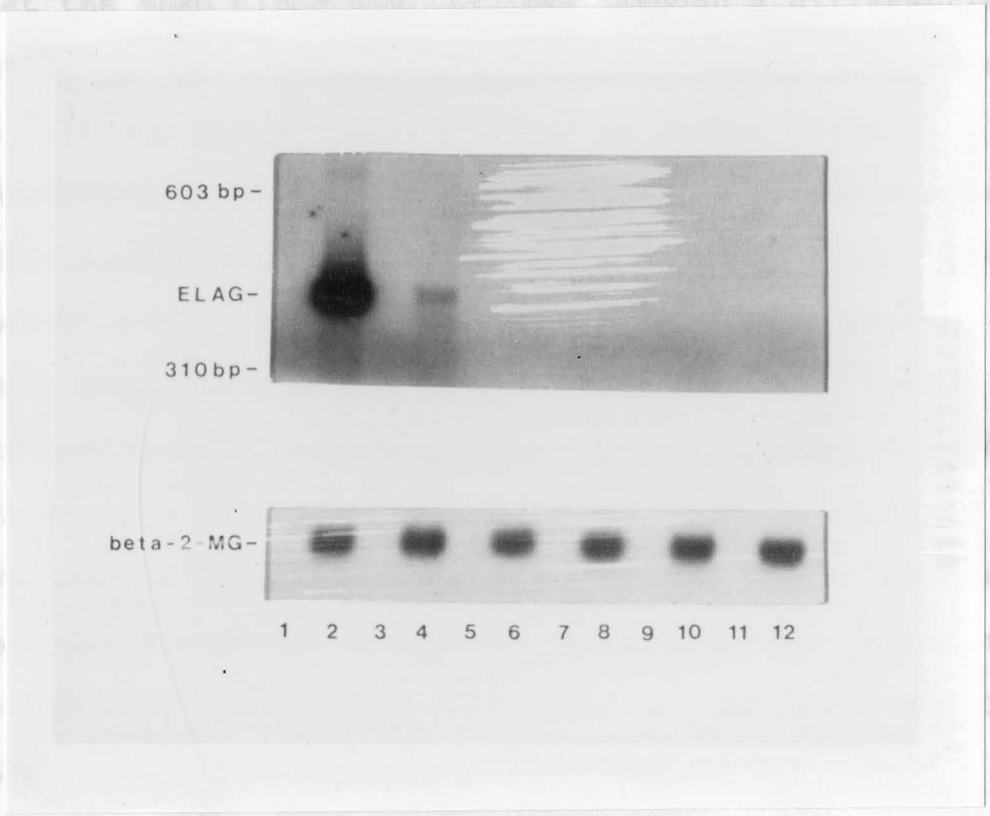

FIGURE 21. Expression of ELAG in human cell lines. RT/PCR results of ELAG and Beta-2-microglobulin expression in L428 Hodgkin's-disease cells (lanes 1 and 2), EBV-infected African Burkitt's lymphoma lines Jiyoye (lanes 3 and 4), P3HR-1 (lanes 5 and 6), and Raji (lanes 7 and 8), the T cell line HuT 78 (lanes 9 and 10) and the normal human fibroblast lin IMR-90 (lanes 11 and 12). cDNA reactions were done with (even lanes) or without (odd lanes) reverse transcriptase. All reactions were supplemented with 1 μCi [α^{32}P]-dATP (3000 Ci/mmol) and the products were resolved by electrophoresis on a 2% agarose gel. Dried gels were exposed to X-ray film at -85°C.

express the highest levels of ELAG (lane 2), while Jiyoye cells express small but detectable amounts of ELAG (lane 4). The P3HR-1 cell line did not express detectable levels of ELAG. I predicted this expression profile given that the ELAG clone was isolated through a differential screening method which targeted genes that are expressed in L428 and Jiyoye cells but not in P3HR-1 cells (described in section I of the Results). Several other cell lines tested do not express detectable levels of ELAG including the EBV infected African Burkitt's lymphoma cell line, Raji (lane 8); the T cell lymphoma line, HuT 78 (lane 10); and the normal lung fibroblast line, IMR-90 (lane 12). This figure is representative of results obtained in four replicate experiments. These results show the restricted nature of ELAG expression in tumor cell lines. Additionally as shown in Figures 11 and 12, ELAG is not expressed in unstimulated Jurkat T cell leukemic line. Further, ELAG expression was not detected in resting PBMC (Figures 8, 9, 10, and 12). Taken together, these results suggest that ELAG is constitutively expressed only in the Hodgkin's-disease cell line L428 and to a lesser degree in the EBV infected African Burkitt's lymphoma cell line Jiyoye.

V. Transcriptional control of ELAG

To understand how aberrant signal could potentially affect ELAG expression in Hodgkin's-disease cells and to understand the molecular basis of the transient expression of ELAG in activated T cells, it was necessary to investigate the signals involved in the regulation of ELAG transcription. To do so, I isolated clones from a WI 38 human lung fibroblast genomic library and sequenced the transcriptional control region. The library was screened using probes containing ELAG sequence regions A and B (see Figures 19 and 20A). Two overlapping clones, designated 6-3 and 6-4 (Figure 22), were isolated. Clone 6-3 contains regions B and C of ELAG 1. Clone 6-4 contains region A of ELAG 1 plus the transcriptional control region. A 4.5-kb *Pst* I fragment of the 6-4 clone was ligated into pBluescript™ and sequenced upstream to position -135, with respect to the designated transcriptional initiation site, using ELAG specific primers. The sequences surrounding the putative transcriptional start site, indicated with an arrow at position +1, are shown in Figure 23. This transcriptional start site was identified by primer extension and 5' RACE (Figures 6 and 7).

No TATA-like element was identified near the expected position of -30 from the transcriptional start site.

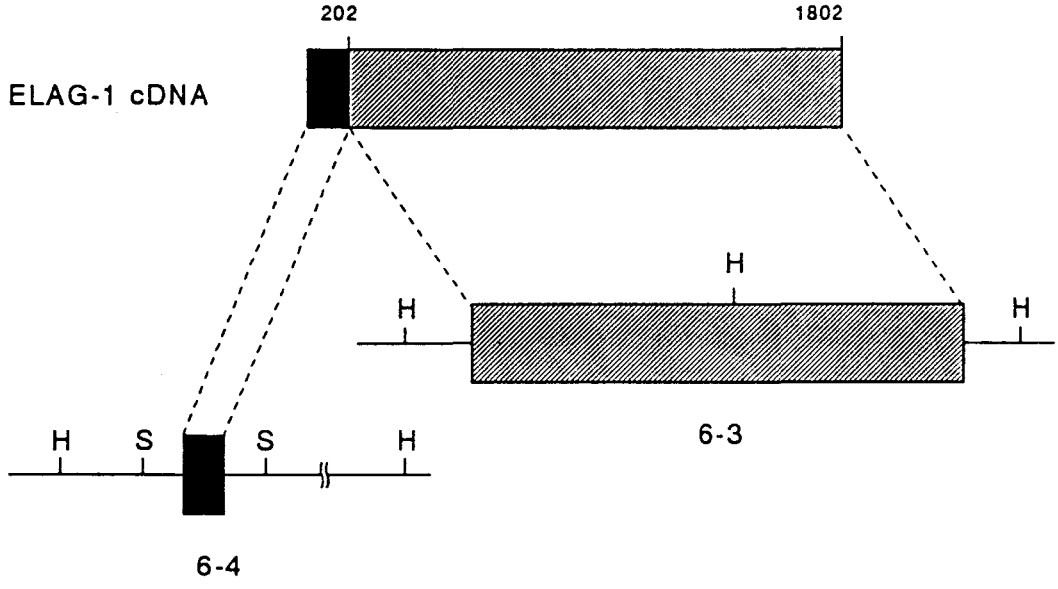

FIGURE 22. Partial restriction map of the overlapping ELAG clones 6-3 and 6-4 which were isolated from the WI-38 human lung fibroblast genomic library. H, *Hind* III; S, *Sac* I.

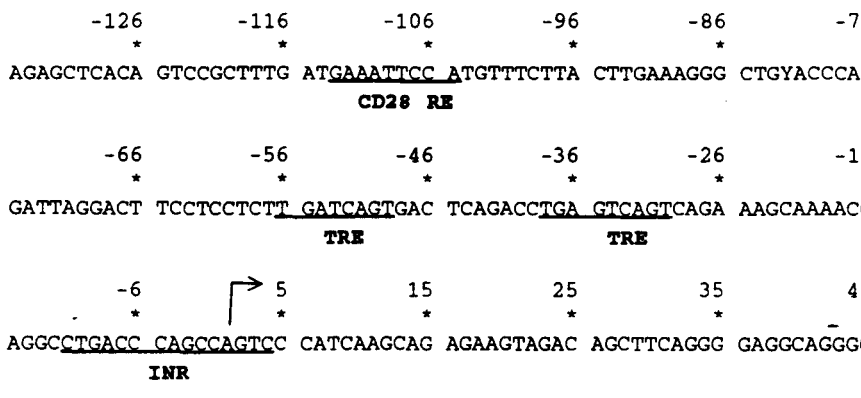

FIGURE 23. Nucleotide sequence of the putative transcriptional control region of the ELAG 1 gene isolated from the genomic library derived from the WI-38 human lung fibroblast cell line. A map of the 135 base pairs immediately 5' of the proposed ELAG transcriptional start site (arrow) contains the INR at position -11 to +4, two tandem TPA response elements (TRE) and a consensus CD28 receptor element (CD28 RE) binding site.

However, sequences surrounding the transcriptional start sited share 80% identity with the initiator region (INR) originally identified in the murine terminal deoxynucleotidyl transferase (TdT) gene (Smale and Baltimore, 1989; Smale *et al.*, 1990). Previous studies have shown that an INR can independently initiate transcription (Smale *et al.*, 1990), therefore, a promoter containing this element does not require a TATA-box. The putative INR at position -11 through +4 likely allows for transcriptional initiation from the adenosine at position +1. However, the primer extension analysis data shows a heterogeneity of transcriptional initiation (Figures 6 and 7). Multiple transcriptional start sites have been described for other genes controlled by TATA-less promoters (Kozmik *et al.*, 1992).

Two TPA response elements (TRE) are aligned at positions -37 and -53 with respect to the transcriptional start site (Holbrook and Fornace, 1993; Angel and Karin, 1991). A consensus CD28 receptor element (Fraser *et al.*, 1991) (CD28 RE) binding site was identified at position -110.

DISCUSSION

Isolation of ELAG: A potential cellular target of EBV immortalization

The novel ELAG (early lymphoid activation gene) cDNA clone was isolated utilizing a differential screening protocol designed to identify genes that are expressed in association with lymphoid cell growth and in the transformation of cells to the Hodgkin's disease phenotype, as well as Epstein-Barr virus (EBV) infected Burkitt's lymphoma. The rationale for this study was based on the recent studies demonstrating by in situ hybridization that the EBV genome is harbored in the Reed-Sternberg cells of diseased tissue in 25-60% of the Hodgkin's disease patients tested (Anagnostopoulos *et al.*, 1989; Weiss *et al.*, 1989; Staal *et al.*, 1989; Libetta *et al.*, 1990). These studies supported a long held speculation that EBV may be a causative agent of Hodgkin's disease. I hypothesized that the oncogenic events responsible for Hodgkin's disease, both EBV associated and EBV negative independent Hodgkin's disease, are mediated by aberrant expression of cellular genes which are also aberrantly expressed in EBV infected non-Hodgkin's lymphoma. My objective was to identify genes involved in

this putative pathway in order to elucidate the signal transduction pathway and biochemical alterations responsible for transformation of cells to the Hodgkin's disease phenotype.

I developed a differential screening procedure (shown in Figure 1) designed to identify genes expressed in association with Hodgkin's disease and that may be cellular targets of the EBV encoded immortalization factor, EBNA 2 (Cohen *et al.*, 1989; Cohen *et al.*, 1992). Clones isolated by this method were tested to determine if they meet three criteria. First, I was interested in novel genes. One gene of interest was the CD30 gene which is expressed following EBV induced immortalization and its expression is associated with Hodgkin's disease. At the time of the screening, the CD30 gene had not been isolated. Second, I was interested in clones that are expressed in association with Hodgkin's disease and EBV mediated transformation. Third, I was interested in clones which are expressed in association with normal lymphoid cells growth, not housekeeping genes. I was interested in such genes because most tumor associated genes (or proto-oncogenes) tend to be involved in the regulation of normal cell growth.

Several clones isolated by the differential screening procedure were sequenced to determine if they met the first criteria (Figures 2 and 3). This study showed that

only one clone demonstrated very low levels of relatedness to any sequences in the GenBank/EMBL data bases (Figure 3), signifying that the clone is likely novel (i.e. previously not described).

Expression of the novel clone was detected in the EBV negative L428 Hodgkin's-disease cell line and in the EBV infected Jiyoye African Burkitt's cell line (Figure 21). These results are consistent with the involvement of ELAG in Hodgkin's disease and Burkitt's lymphoma. Additionally, ELAG was not expressed at detectable levels in the P3HR-1 African Burkitt's lymphoma cell line. This was expected in that clones isolated by differential screening were selected based on their expression in Jiyoye but not P3HR-1 cells. Jiyoye cells are infected with a viral strain containing EBNA 2, and the phenotype of these cells as indicated by the expression of cell surface proteins suggest that EBNA 2 has induced the expression of cellular genes associated with EBV mediated immortalization. P3HR-1 cells, on the other hand, are infected with and EBNA 2 negative, non-immortalizing strain of EBV. The cellular environment in which ELAG is expressed indicates that it is a potential target of EBNA 2, either directly or indirectly. EBNA 2 is a transactivator of cellular genes including CD23 (Wang *et al.*, 1990; Wang *et al.*, 1987). It would be interesting to

determine if cis elements in the transcriptional control region of ELAG are substrates of EBNA 2 activity.

The role of ELAG in normal T cell activation

A third criteria that I established to determine if clones isolated by differential screening were suitable for additional study was that they should be expressed in association with normal cell growth and proliferation. I was interested in identifying genes which could potentially be involved in the development of the transformed phenotype of Hodgkin's disease. Transformation associated genes (i.e. proto-oncogenes) tend to function normally in the regulation of cell growth and proliferation (Bishop, 1987). Therefore, if ELAG is involved in the oncogenesis of Hodgkin's disease, it would likely be involved in the regulation of normal cell growth. During a study designed to determine the role of ELAG in normal cell growth I demonstrated that the novel clone is expressed in association with the activation of T lymphocytes in a population of peripheral blood mononuclear cells (PBMC; Figures 8, 9, 10 and 12).

The novel ELAG gene is expressed within one hour following activation of PBMC with anti-CD3 monoclonal antibody (Figure 10). This antibody specifically stimulates T cell activation by binding to the T cell receptor/CD3 molecule, also known as the antigen receptor

complex. These data strongly suggest that ELAG is expressed in activated T cells of the PBMC population. To help confirm this observation, ELAG expression was studied in Jurkat human leukemic cell line which is an accepted tissue culture model for the investigation of events during T cell activation. Jurkat cells can be stimulated to produce IL-2 by mechanisms that are analogous to those in activated T cells (Gillis and Watson, 1980). Activation of Jurkat cells requires two signals, stimulation of the TCR/CD3 complex coupled with induced activation of protein kinase C (Gillis and Watson, 1980). In tissue culture, these signals can be provided by the addition of PHA (phytohemagglutinin) and PMA (phorbol 12-myristate 13-acetate, also known as TPA). I found that *in vitro* activation of Jurkat cells with PMA and PHA will induce ELAG expression in four hours (Figure 11). These data are consistent with the designation of ELAG as a T cell activation gene. Furthermore, either of these two signals added in culture individually is sufficient to induce ELAG expression. In contrast, expression of IL-2 requires two signals; both PHA and PMA (Gillis and Watson, 1980). These data indicated the biochemical signals required for induction of ELAG expression are less complex than the signals required for stimulation of IL-2 production. Additionally, the AP-1 transcription factor is activated by stimulation with PMA (Holbrook and

Fornace, 1993; Angel and Karin, 1991). Induction of ELAG expression with PMA alone further supports the argument that ELAG is an immediate downstream target of AP-1 transcriptional activator.

T cell activation is very complex and is poorly understood. More than 100 different genes and proteins have been identified that are expressed during T cell activation (Crabtree, 1989; Altman *et al.*, 1990), and their expression is activated in a coordinated manner that is tightly regulated (Altman *et al.*, 1990; Ullman *et al.*, 1990; Furue *et al.*, 1990). The complexity of this cascade of genes is highlighted by the recent studies of Zipfel *et al.* who have isolated 60 novel cDNA clones, by subtractive screening, which are expressed following T cell activation (Zipfel *et al.*, 1990; Irving *et al.*, 1989). These investigations support the contention that there are many signals involved in T cell activation that are not understood. Because ELAG is expressed in activated T cells, further study of its properties may provide additional information about the molecular events required in the immune response and in general in lymphoid cell growth and proliferation.

Crabtree has categorized the cascade of genes expressed during T cell activation into three major groups (Crabtree, 1989; Ullman *et al.*, 1990); immediate early activation genes, early activation genes and late

activation genes. Immediate early genes are expressed in the first 30 minutes following activation and include c-*fos* (Distel and Spiegelman, 1990; Grausz *et al.*, 1986; Moore *et al.*, 1986; Gonda and Metcalf, 1984), c-*myc* (Reed *et al.*, 1986; Grausz *et al.*, 1986; Moore *et al.*, 1986; Reed *et al.*, 1985; Gonda and Metcalf, 1984), and NF-κB (Jamieson *et al.*, 1991). These genes generally are involved in transcriptional activation. The second group of genes expressed between thirty minutes and forty-eight hours are known as the early activation genes. Genes expressed after 48 hours are called the late activation genes.

Based on my studies, ELAG is best categorized as an early antigen, induced to expression by stimulation with anti-CD3 antibody within one hour but is no longer detectable by twelve hours post-activation (Figures 9 and 10). Included in the group of early activation genes are the IL-2 receptor (Wakasugi *et al.*, 1985; Leonard *et al.*, 1984; Nikaido *et al.*, 1984), IL-2 and other interleukins (Norma, 1986; Kinashi *et al.*, 1986; Hirano *et al.*, 1986), and c-*myb* (Reed *et al.*, 1986; Stern and Smith, 1986; Torelli *et al.*, 1985; Torelli *et al.*, 1985; Thompson *et al.*, 1986). The early genes are heterogeneous in their function and are expressed prior to DNA synthesis. Therefore, they probably act as mediators between the immediate early genes and the signals that actually induce

DNA synthesis and cell division (Altman *et al.*, 1990). Analysis of the transcriptional control region of ELAG shows that it contains two TPA Response Elements (TRE; Figure 23). The TRE is a consensus binding site for the AP-1 transcription factor. AP-1 factor is a heteroduplex of Jun and Fos proteins. Expression of Fos is sharply increased during T cell activation (Distel and Spiegelman, 1990; Grausz *et al.*, 1986; Moore *et al.*, 1986; Gonda and Metcalf, 1984) prior to the expression of ELAG. The presence of TRE elements in the ELAG promoter region, and the temporal relationship between the expression of ELAG and Fos strongly suggest that ELAG is an immediate downstream substrate for the AP-1 factor. The effect of AP-1 activity during T cell activation is poorly understood because, to date, very few genes expressed during T cell activation have been shown to contain a TRE. However, it is thought that AP-1 is a critical nuclear factor required to induce expression of other cellular genes that are necessary to regulate the T cell activation cascade.

The molecular response required to commit to the activation cascade is made between 30 minutes and 2 hours. The signals required for this event are poorly understood. ELAG is expressed in a time that might allow it to provide an important signal required for the T cell to commit to the activation pathway. Additionally, ELAG is expressed

during a period when the activated T cell advances from G_0 to G_1 in the cell cycle. IL-2, which is expressed between 1 and 24 hours following T cell activation, has been shown to be essential for inducing the T cells to enter cell division. Similar signal requirements and similar temporal expression indicate that ELAG may also be directly involved in lymphoid cell proliferation.

ELAG potentially encodes a nuclear phosphoprotein

To better understand how ELAG might function during T cell activation and possibly in the regulation of cellular proliferation, I analyzed the predicted open reading frame to identify structural motifs.

I have shown by in vitro translation that the translation of a 13.2-kDa protein is initiated from a CUG translational start site (Figure 16). Very few eukaryotic genes identified encode non-AUG start sites but the examples are well documented. The concept that all mammalian cellular proteins were translated from an AUG initiation codon was dispelled when Pirastu *et al.* showed that a human alpha-globin gene containing a mutated start codon still allowed for the production of a protein; although this mutation is associated with a clinical thalassemia (Pirastu *et al.*, 1984). This finding demonstrated that the cells translational machinery could recognize a non-AUG translational initiation site. Since

then, several normal genes have been identified which encode proteins that initiate from non-AUG codons including the Drosophila encoded choline acetyltransferase (Sugihara *et al.*, 1990), viral encoded genes including the murine leukemia virus gp85^{gag} (Prats *et al.*, 1989), equine infectious anemia virus Tat and Rev proteins (Stephens *et al.*, 1990), and the parainfluenza virus C' protein (Prats *et al.*, 1989). Several human genes utilize non-AUG translational initiation sites and include basic fibroblast growth factor (Prats *et al.*, 1998; Clements *et al.*, 1988), choline acetyltransferase (Strauss et al., 1991), c-*myc* (Hann *et al.*, 1992; Hann *et al.*, 1988), *int*-2 (Acland *et al.*, 1990), *TEF*-1 (Xiao et al., 1991), *Itk* (Bernards and de la Monte, 1990), *Hck* (Lock *et al.*, 1991), *Krox*-24 (Lemaire *et al.*, 1990), and *Trk* (Martin-Zanca *et al.*, 1989).

The role of non-AUG translational initiation sites is unclear; however, recent investigations have shown that multiple isoforms of *int*-2 which are translated from the same gene are localized to different sub-cellular compartments (Acland *et al.*, 1990). Additionally, Prats *et al.* suspect that the use of a non-AUG start site may be a post-transcriptional control mechanism (Prats *et al.*, 1998).

Computer analysis predicts the presence of an amino acid motif beginning at position 2 (Figure 13), Pro-Arg-

Ala-Lys-Arg, that is similar to nuclear localization signal (NLS) motifs identified in known nuclear factors (Dingwall and Laskey, 1986). This property suggests that ELAG is likely to function in the nucleus.

An NLS is the minimum amino acid sequence required to transport protein to or retain protein in the nucleus. The best characterized NLS is encoded by the SV40 large T antigen. The minimum sequence of amino acids required for localization of T-antigen in the nucleus is Pro-Lys-Lys-Lys-Arg-Lys-Val (Lanford and Butel, 1984; Kalderon *et al.*, 1984; Kalderon *et al.*, 1984). Since the identification of this motif, similar, yet non-identical, signals have been identified in other proteins (Dingwall and Laskey, 1986). The function of the individual amino acids in the NLS is unclear, but they are probably important in recognition by chaperone proteins.

The NLS is likely to be involved in the transportation of large proteins into the nucleus. Proteins greater than sixty kilodaltons (kDa) are excluded from diffusing through the nuclear pore where small proteins freely diffuse (Paine, 1975). However, smaller proteins diffuse out at the same rate in which they diffuse into the nucleus. Therefore, they must be selectively retained (Feldherr and Ogburn, 1980; Merriam and Hill, 1976). It is likely that the NLS serves this purpose for proteins smaller than 60-kDa. Since ELAG is

only 13.2-kDa, the putative NLS may be important for retaining the protein in the nucleus.

Also identified in the predicted amino acid sequence is a $Cx_3Cx_{12}Hx_2H$ (cysteine followed by 3 amino acids, another cysteine followed by 12 amino acids, a histidine followed by 2 amino acids and a final histidine) motif similar to the tandemly-repeated pattern identified in the amino acid sequence of the Xenopus TFIIIA zinc finger protein (Figure 13)(Miller *et al.*, 1985; Brown *et al.*, 1985). Zinc fingers are protein domains that fold producing an alpha helical structure which binds a zinc ion.

Hundreds of genes identified encode a zinc finger-like motif similar to the zinc finger motifs of the TFIIIA transcription factor (Miller *et al.*, 1985; Brown *et al.*, 1985; Klevit, 1991). The function for most of these putative zinc finger containing proteins in many cases is not known. However, several proteins definitively shown to encode a zinc finger domain are well characterized and serve important regulatory functions including the regulation of gene expression including growth signals EGR1 (Early Growth Response gene 1)(Sukhatme *et al.*, 1988) and EGR2 (Early Growth Response gene 2)(Joseph *et al.*, 1988), Drosophila segmentation genes *Hunchback* (Tautz *et al.*, 1987), and *Kruppel* (Rosenberg *et al.*, 1986). In addition to these examples, several recently identified

genes involved in T cell development and activation encode zinc finger proteins including *Ikaros* (Georgopoulos *et al.*, 1992) and pAT133 (Muller *et al.*, 1991).

Zinc finger containing proteins have been primarily identified as transcriptional regulators due to their specificity for binding target DNA in the promoter region of certain genes. Known transcriptional factors that utilize the zinc finger structure which directly interact with DNA include TFIID and ZIF268. These proteins interact with different cis elements showing that all zinc fingers are not alike. However, some studies show that the zinc finger region binding to DNA requires two guanosine residues with the appropriate spacing (Pavletich and Pabo, 1991). The finger motif binds in the large groove of the target DNA sequence (Pavletich and Pabo, 1991). Most zinc finger encoding proteins contain multiple finger motifs, however, only a single zinc finger domain is required for DNA binding (Baldarelli *et al.*, 1988; Lee *et al.*, 1989; Parrage *et al.*, 1988)

Zinc finger domains have functions other than transcriptional regulation through DNA binding. TFIIIA from Xenopus has been shown to bind to both RNA and DNA (Darby and Joho, 1992; Clemens *et al.*, 1993). Recently Gottesfeld *et al.* molecularly dissected the TFIIIA protein, which encodes nine zinc finger domains, to determine which domains were essential for DNA binding and

those essential for RNA binding. Their results demonstrate that some zinc fingers were only involved in RNA binding while the others were involved with DNA binding (Clemens *et al.*, 1993). However, they were unable to identify any properties such as a consensus sequence which would distinguish a DNA-binding zinc finger domain from an RNA-binding zinc finger domain.

Other evidence suggests that zinc fingers may be involved in protein-protein interactions. The DNA binding domain of the estrogen receptor encodes two zinc finger-like motifs; one has DNA binding capacity, the other has a protein interaction ability that allows it to dimerize with other estrogen receptor molecules (Umesoso *et al.*, 1991). This protein interaction which allows for the dimerization is essential for stable DNA binding (Umesoso *et al.*, 1991).

Computer analysis predicts that four amino acids are in the correct sequence context to be phosphorylated (Figure 13). Phosphorylation is a regulatory mechanism that biochemically alters a protein. Such a biochemical modification often functions as an on/off switch, changing the protein from an inert state to an active one or vice versa. Three amino acids Ser-48, Ser-62 and Thr-102 are potential substrates for phosphorylation by protein kinase C, a ubiquitous enzyme involved in the regulation of several signal transduction pathways. Ser-9 is a

potential substrate for c-*raf*, a proto-oncogene involved with a signal transduction pathway that is associated with cellular immortalization.

The predicted structural properties suggest that ELAG may be a nuclear phosphoprotein. Further, the zinc finger motif would provide the capability to directly affect expression of other genes through protein/nucleic acid interactions. Direct analysis of the phosphorylation state of the ELAG protein may be critical in understanding how the protein activity is regulated. Phosphorylation/ dephosphorylation is an important cellular mechanism involved in the regulation of protein activity and localization (Bishop, 1987). Given the temporal characteristics of ELAG expression, I hypothesize that ELAG provides a signal to the nucleus that continues the cascade of T cell activation genes and initiates the transition from G_0 to G_1 of the cell cycle.

Is ELAG a novel oncogene?

ELAG was isolated in an effort to identify novel oncogenes that are involved in the tumorigenic transformation of cells Hodgkin's disease and EBV infected Burkitt's lymphoma. Two major mechanisms are involved in activating oncogenes (Bishop, 1987); 1) increased expression in oncogenes such as *myc* and 2) mutationally altered structure/function of the encoded protein like

Ras. To address the possibility that a mutation of ELAG is a transformation specific event, I was interested in comparing the sequence of ELAG cDNA isolated from activated PBMC versus the sequence of ELAG cDNA isolated from the L428 cell line. Only ELAG 3 type clones were isolated from activated PBMCs. A comparison with the normal clone shows that L428 derived ELAG 3 does not encode any mutations in the cDNA (Figure 18). Therefore, the potential role of ELAG 3 in transformation would only be due to constitutive or aberrant expression.

During this investigation I found that ELAG is expressed as a family of at least four transcripts in the L428 Hodgkin's-disease cell line. Sequence analysis showed that ELAG clones 1, 2, and 3 share the same open reading frame that encodes a putative 13.2-kDa protein but have different 3' untranslated termini. ELAG 4 would encode the same amino terminus as the other 3 ELAG clones but because of a shortened open reading frame the entire protein would be only 8.2-kDa and ELAG 4 retains the putative NLS but would not encode the zinc finger motif. The role of the various transcripts and encoded proteins is unknown. These individual transcripts might be expressed in the normal counterpart of the Hodgkin's cell, which as of yet has not been definitively described. The various transcripts are potentially expressed in normal tissues other than activated T cells. To address this

possibility, we will have to study ELAG expression in an expanded panel of cells and tissue types. If this were the case one might suggest that expression of all four isotypes in the L428 cell is potentially a tumor specific event. To date, ELAG 4 has not been identified in normal activated PBMC. Aberrant expression of the ELAG 4 encoded protein may represent a tumor specific event. If this were the case, one might speculate that the ELAG 4 has a counter acting effect of a zinc finger-encoding ELAG protein, and that this counter activity is a critical deregulatory event that allows that cell to proliferate.

Because ELAG expression is tightly regulated during T cell activation, it is a good candidate gene that when aberrantly expressed could contribute to the transformed phenotype of Hodgkin's disease. To test this possibility, ELAG expression was analyzed in several tissue culture lines. The data showed that ELAG is expressed at the greatest levels in the Hodgkin's-disease cell line L428. Expression was also detected in the EBV infected Burkitt's lymphoma cell line Jiyoye but not in P3HR-1, the Jiyoye derivative. The Jiyoye cell line is infected with a fully transforming virus that encodes EBNA 2 (Hinuma and Grace, 1967), a protein that is necessary for the ability of the virus to immortalize cells. In contrast, P3HR-1 is infected with a virus which does not express the EBNA 2 gene due to a deletion in the viral genome and therefore

is non-transforming (Jeang and Hayward, 1983; Bornkamm *et al.*, 1982). Consequently, ELAG may be expressed in association with a signal transduction pathway that is involved in Hodgkin's disease as well as transformation by EBV. In addition to the analysis of these cell lines, I showed that ELAG is not expressed at detectable levels in the African Burkitt's lymphoma cell line Raji, the T cell lymphoma line HuT 78, the cervical carcinoma line HeLa, or the normal lung fibroblast line IMR-90. In addition, ELAG expression was not detectable in unstimulated Jurkat leukemic T cell line (Figure 11) or in unstimulated peripheral blood mononuclear cells (Figures 8, 9, 10 and 12). These results suggest that ELAG is very tightly regulated in most cells but is constitutively expressed in the Hodgkin's-disease cell line L428 and the African Burkitt's lymphoma cell line Jiyoye. This deregulated pattern of expression may be associated with the transformed phenotype of Hodgkin's disease and EBV transformed cells.

Transcriptional control of ELAG

The transcriptional control region of ELAG was sequenced in an effort to understand how aberrant signal could potentially affect ELAG expression in Hodgkin's-disease cells and also, to investigate the molecular basis of the transient expression of ELAG in activated T cells.

ELAG has a TATA-less promoter (Figure 23) which contains an INR that is 80% identical to the INR described in the murine TdT gene (Suzow and Friedman, 1993; Smale *et al.*, 1990). INR control elements tend to be associated with tightly regulated genes involved in activation, differentiation and development (Suzow and Friedman, 1993). This is consistent with the tight regulation of ELAG that is observed in activated T cells.

Previous studies have shown that TATA-less genes have a heterogeneous transcriptional initiation site (Kozmik *et al.*, 1992). Primer extension analysis showed multiple banding near the region that is designated as +1 (Figures 6 and 7) indicating a heterogeneity of transcriptional initiation. It is likely that the TATA element found in most promoters is involved in ensuring that the transcriptional complex initiates from a single site, however initiation is more variable in the absence of a TATA element (Kozmik *et al.*, 1992).

One trans-acting signal that is likely to be involved in the transcription of ELAG is the Jun/Fos AP-1 complex. This complex functions by binding to the TPA response element (TRE) and is involved in transactivating several genes in response to extracellular stimulus (Holbrook and Fornace, 1993; Angel and Karin, 1991). PMA stimulation of Jurkat cells, which activates AP-1 activity, induces ELAG expression by four hours (Figure 11). Additionally, I

have identified a tandem of TREs at positions -37 and -53 (Figure 23), providing strong evidence that the AP-1 complex is involved in regulation of ELAG expression. Few early activation genes have been identified that have a TRE, thus ELAG may represent one of the few early activation genes that may be a substrate for the AP-1 complex. Promoters containing multiple TRE elements have been shown to be induced to higher levels than promoters containing only a single element and therefore the role of AP-1 on ELAG expression may be very important.

A consensus sequence for the binding of the CD28 receptor element (CD28 RE) was identified at position -110 from the designated transcriptional start site (Figure 23). Recent studies have shown that CD28 is an important cell surface molecule that transduces a costimulatory signal from an antigen presenting cell that is likely to be required for T cell activation (Shaninian *et al.*, 1993). CD28 then transactivates cellular genes by inducing the activity of a nuclear DNA binding protein (Fraser *et al.*, 1991). Genes stimulated through the CD28 RE include IL-2 (Fraser *et al.*, 1991), IL-3, GM-CSF, and interferon-gamma (Fraser and Weiss, 1992). Definitive evidence that stimulation of the CD28 RE will augment ELAG transcription would provide strong support for the notion that ELAG is essential for T cell activation.

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VITA

The author, John S. Bennett, was born in Elmhurst, Illinois on October 23, 1964, to Robert and Lynne Bennett.

In August 1982, Mr. Bennett entered the University of Wisconsin - Stevens Point, in Stevens Point, Wisconsin, and received the degree of Bachelor of Science in both Biology and Chemistry in 1987. That year he enrolled in the Program in Molecular Biology at Loyola University Medical Center, Maywood, Illinois. In the Spring of 1988, He joined the laboratory of John F. Nawrocki, Ph.D., where he initiated studies to characterize genes involved in the tumorigenic transformation of cells in Hodgkin's disease. In 1991, Mr. Bennett was awarded a University Fellowship.

Mr. Bennett has accepted a position as a post-doctoral fellow in the laboratory of Kathleen Rundell, Ph.D. at Northwestern University, Chicago, Illinois. His studies will focus on the role of Simian virus 40 small-t antigen in tumorigenic transformation.

APPROVAL SHEET

The dissertation submitted by John Scott Bennett has been read and approved by the following committee:

John F. Nawrocki, Ph.D.
Assistant Professor
Department of Pathology
Department of Immunology and Microbiology
Loyola University Medical Center

Leonard C. Erickson, Ph.D.
Director, Program in Molecular Biology
Professor
Department of Medicine
Loyola University Medical Center

Cecilia A. Hofmann, Ph.D.
Professor
Department of Molecular and Cellular Biochemistry
Loyola University Medical Center

Mark R. Kelley, Ph.D.
Associate Professor
Department of Pediatrics
Indiana University School of Medicine

Katherine L. Knight, Ph.D.
Professor and Chairperson
Department of Immunology and Microbiology
Loyola University Medical Center

Mary Manteuffel, Ph.D.
Professor
Department of Molecular and Cellular Biochemistry
Loyola University Medical Center

The final copies have been examined by the director of the dissertation and the signature which appears below verifies the fact that any necessary changes have been incorporated and that the dissertation is now given final approval by the Committee with reference to content and form.

The dissertation is therefore accepted in partial fulfillment of the requirement for the degree of Doctor of Philosophy.

December 1993 John F. Nawrocki, Ph.D.

Date Director's Signature